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INEFFECTIVENESS OF ADRENAL CORTEX PREPARATIONS IN THE TREATMENT OF EXPERIMENTAL SHOCK IN NON-ADRENALECTOMIZED DOGS[1]

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The conclusion that the adrenal plays an important rôle in increasing the resistance of the organism to shock is largely derived from experiments made on adrenalectomized animals. The animal deprived of adrenals is extremely sensitive to minor stress procedures, and rapidly develops circulatory failure unless cortical extract or certain adrenal steroids are promptly administered. Whether the circulatory failure of adrenalectomized animals and shock in intact animals are due to the same functional deficiency remains to be proven. The assumption that because cortical extract will raise the resistance of the adrenalectomized animal to that of the normal, it will also prove beneficial in the treatment of shock, presupposes that the glands of the shocked intact animal are no longer able to secrete sufficient adrenal hormones to meet body requirements. At present such an assumption lacks definite experimental proof.

The available evidence concerning the therapeutic value of adrenal cortical hormones in shocked intact animals is highly conflicting. For example, Heuer and Andrus (1) found that when cortical extract was added to a plasma transfusion, the shock which follows the injection of the contents of a closed intestinal loop could be prevented. Likewise, Wohl, Burns and Pfeiffer (2) observed a beneficial effect of cortical extract, when given with infusions of physiological salt solution, in cases of high intestinal obstruction. However, Fine, Fuchs and Mark (3) reported that while desoxycorticosterone acetate (DCA) prevented the plasma volume decrease which occurs after intestinal obstruction, it had no

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Princeton University.

[2] Some of the data included in this paper were taken from a thesis presented by R. R. Overman to the Graduate School of Princeton University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] Upjohn Research Fellow.

clear effect in preventing fatal shock. Helfrich, Cassels and Cole (4) found that cortical extract administered to dogs before shock was induced by intestinal manipulation, greatly retarded the blood pressure decline. The same workers state that in experimental shock produced by hemorrhage, cortical extract exerted a definite but slight tendency to decrease the severity of the shock, particularly when the extract was given in conjunction with glucose and electrolytes. Fine and co-workers (5), however, have recently reported inconclusive results with the use of extract following hemorrhage, and Huizenga *et al.* (6) could find no evidence of a beneficial effect of extract in carefully controlled hemorrhage experiments.

Cortical extract has been used in anaphylactic shock in guinea pigs (7) and dogs (8), with positive results in the former and negative in the latter case. Perla and co-workers (9) claimed that cortical extract plus saline protected rats against toxic doses of histamine more regularly than would the use of saline alone.

With regard to traumatic shock, Weil, Rose and Browne (10) found a marked reduction in the mortality of rabbits subjected to visceral manipulation when cortical extract was given, but no such effect was observed with DCA. Likewise, Selye and co-workers (11) found cortical extract and certain adrenal steroids to be effective after intestinal trauma in rats, but DCA was actually harmful. Noble and Collip (12) found a slight increase in the resistance of rats to the generalized trauma produced by whirling the animal in a specially designed drum. Wilson and Stewart (13) found that although DCA prevented the decline in plasma electrolyte concentrations following scalds, it did not prevent fatal shock. However, Katz and his associates report that DCA is highly effective in preventing shock in dogs subjected to venous occlusion (14). Cortical extract, while less potent in this respect, definitely showed a positive effect (15). Treatment of the shocked human patient with extract and adrenal steroids has not, in general, given clear cut results and the efficacy of such treatment is in dispute (9, 16–22).

Evidence for the therapeutic value of adrenal hormones would seem to depend on the selection of a shock inducing procedure which could be standardized, and would yield a shock condition which satisfied three basic requirements: a. The shock should terminate in death in nearly all untreated control animals, in an interval of some 4–24 hours. On the other hand, the survival period should be long enough to allow injected cortical extract or steroids sufficient time to exert full effect. b. The trauma administered should not result in such mutilation as to necessitate the animal being kept under anesthesia throughout the experiment. c. The shock involved should be reversible, and hence responsive to therapeutic measures. We have studied three shock inducing procedures which seem to satisfy these criteria for testing adrenal cortical extract or steroid therapy.

I. *Shock following release of leg tourniquets.* The release of tight tourniquets applied to both hind legs of a dog, after a 5 hour period of constriction, is followed by fatal shock in 96 per cent of untreated control dogs (23).[4] The initiating

[4] In a previous tourniquet paper, This Journal **138:** 156, 1942, the diameter of tubing employed is erroneously given as 120 mms. This should be 12. mms.

factor seems to be the rapid and extensive leaking of plasma through the capillaries into the tissues of the injured legs. In the first hour after tourniquet release, and before clinical signs of shock are apparent, there is a 40 per cent rise in hematocrit and hemoglobin levels, and a proportional decrease in plasma volume as measured by T-1824 dye technique (24). Shock, as evidenced by a mean blood pressure level of 75–80 mm. Hg, a decline in skin temperature, pallid mucous membranes and gums, and a lessening of the need for nembutal anesthesia, follows within 3–4 hours. The survival periods of control animals subjected to constriction of both hind legs for 5 hours ranged from 3–27 hours, the average being 8 hours.

The extract used in the experiments was a concentrate prepared in this laboratory (1 cc. = 120 grams fresh adrenal tissue) with activity such that 1–2 cc. daily was sufficient to maintain a 10 kgm. adrenalectomized dog in good health and vigor with normal serum electrolytes, blood glucose and blood pressure.

It was recognized that single intravenous injections of cortical extract into an animal which was losing its plasma volume so rapidly into the injured legs, perhaps could not provide a blood hormone level constant or high enough to restore the damaged vascular bed. For example, the administration of large amounts of physiological salt solution by a single injection is followed by immediate swelling of the legs, with little discernible interruption of the rise in hematocrit level or amelioration of the shock symptoms (23). Even when a total of 50 cc. of extract was given in small doses, intravenously or intra-arterially, over a period of hours, it did not retard the development of shock, reduce the hemoconcentration, or prevent death.

The injection technique was therefore modified so that a constant source of extract was available to the animal after release of the leg tourniquets. A 20 gauge needle was connected to a sterile reservoir maintained at constant temperature, and immediately upon release of the constrictions 280 cc. of cortical extract allowed to drip into the jugular vein at a rate slightly greater than 1 cc. a minute. There was no evidence that the cortical extract so given modified the degree of hemoconcentration or the usual sequence of events leading to fatal shock (table 1, dog 1).

Death following release of leg tourniquets can be prevented by wrapping the injured legs in tight bandages, covered with adhesive tape, immediately following tourniquet release (23). This procedure reduces the volume of plasma lost into the legs, and hence decreases the hemoconcentration and decline in plasma volume. If these bandages are allowed to remain for a sufficient length of time, the capillaries of the legs recover and circulation is restored. The bandages can then be removed without a marked increase in hemoconcentration and development of fatal shock. The critical period for rehabilitation of the capillaries would seem to lie between 18 and 24 hours. At least, when the bandages were removed at the end of 24 hours, 2 of 4 dogs showed a subsequent extreme hemoconcentration and died in shock, and the other 2 exhibited but moderate blood concentration, followed by a slow hemodilution, and eventually recovered. An attempt to remove the bandages at the end of 18 hours in 3 additional dogs was followed by fatal shock in every case.

The bandaging technique seemed ideally suited to a crucial test of the therapeutic value of cortical extract, since it lengthened the time over which administered hormones could exert an influence. The patency of the circulation in the bandaged limbs was shown by injecting the dye T-1824 into the saphenous vein, and recovering it within a few seconds from the jugular. A total of 50

TABLE 1

Non-effectiveness of cortical extract and of desoxycorticosterone acetate in the treatment of shock following release of leg tourniquets and following muscle trauma in intact dogs

TIME	BLOOD PRESSURE	PULSE	HEMATOCRIT	HEMOGLOBIN	REMARKS
Dog 1. 10.2 kgm. Tourniquet shock, given 280 cc. cortical extract					
	mm. Hg	*per minute*	*per cent*	*grams per cent*	
10:15 a.m.	108	130	42.1	15.3	Tourniquet applied to both hind legs
3:15 p.m.	110	166	46.0	16.3	Tourniquets released. Started infusion of cortical extract by intravenous drip
7:15 p.m.	75	180	71.2	29.3	Infusion completed. Dog died at 11 p.m.
Dog 2. 13.6 kgm. Tourniquet shock, 18 hour leg bandaging, given 50 cc. cortical extract					
8:00 p.m.	108	104	32.8	12.6	Tourniquets released after 5 hr. constriction period, tight bandages applied. Given 10 cc. cortical extract intravenously at 9 p.m., 11 p.m., 8 a.m., 11 a.m. and at 2 p.m. when bandages removed
6:30 p.m.	67	190	66.7	19.8	Dog died at 7 p.m.
Dog 3. 6.1 kgm. Tourniquet shock, given 5 transfusions of 3.1 cc. plasma concentrate each, and 5 injections of 6.2 cc. cortical extract each					
3:00 p.m.	100	144	37.9	13.8	Tourniquets released, first transfusion
4:00 p.m.	62	80	59.1	21.4	Second transfusion
5:00 p.m.	68	128	56.7	22.3	Third transfusion
7:00 p.m.	63	184	60.0	22.3	Fourth transfusion
10:00 p.m.	62	172	63.6	23.4	Fifth transfusion. Dog died at 11 p.m.
Dog 4. 6.2 kgm. Muscle trauma shock, given 70 mgm. DCA fore-treatment and 75 cc. cortical extract					
9:45 a.m.	103	120	44.0	17.3	Trauma of 1000 blows completed at 10:15 a.m. Extract in 10 cc. doses given half-hourly
4:00 p.m.	32	208	38.8	17.5	Dog died at 4:25 p.m.

cc. of extract, in divided doses, was then given to each of 3 dogs whose hind legs were tightly encased in bandages. When the bandages were removed at the end of 18 hours, the legs swelled, the blood concentrated, and death from shock ensued (table 1, dog 2). It would seem that the injected extract had neither prevented shock nor hastened repair of the capillary bed in the injured legs.

The steroid DCA[5] was also used on a series of dogs with legs bandaged for 18 hours after release of tourniquets. Since this hormone was in oil, repeated injections were made intramuscularly over the 24 hours preceding the experiment as a prophylactic treatment. The results were similar to those where cortical extract was employed, since as much as 50 mgm. DCA did not prevent shock or reduce the hemoconcentration when the bandages were removed.

The possibility remained that cortical extract might be effective against this relatively severe type of shock only if given in conjunction with a plasma transfusion. A transfusion of 25 cc. of dog plasma per kilogram body weight could prevent death after release of the leg tourniquets, only when given in a certain time sequence (23). A single transfusion, whether given immediately upon release of the tourniquets or later when the animal was already in shock, prevented death in only a few cases. When, however, the same volume of plasma was divided into 5 equal portions, and injected at the time the tourniquets were released, and at the end of the 1st, 2nd, 4th and 7th hours thereafter, fatal shock was prevented in all cases tested.

The relative ineffectiveness of the single transfusion might be explained by assuming that the rapid expansion of the circulating plasma volume had increased the rate of plasma transfer through the damaged capillaries, so that little permanent benefit would result. To test this point, a plasma protein concentrate was made by dialyzing freshly prepared dog plasma until free of salts, concentrating it to a third its original volume under reduced pressure at low temperature, and adding NaCl to 0.9 per cent. A single injection of 5 cc. per kgm. body weight of this concentrate could not prevent death in 4 dogs following release of tourniquets. When the 5 cc. per kgm. was divided into 5 injections of 1 cc. per kgm. each, and given intermittently over a 7 hour period, fatal shock was prevented in 4 of 5 dogs. It seems obvious that the difference in the efficacy of the two transfusion procedures was not merely a reflection of the volume administered, but depended on the time sequence with which they were given.

The amount of plasma concentrate, administered in 5 equal doses, was then step-wise reduced until fatal shock could no longer be prevented. For instance, a total of 3.5 cc. per kgm. body weight allowed successful recovery of 2 of 3 dogs. A dose of 2.5 cc. per kgm. did not prevent death in 3 additional animals. Thirty-five cubic centimeters of the cortical extract was then given in conjunction with a transfusion of 2.5 cc. kgm. body weight in 2 dogs. Both showed hemoconcentration changes similar to those of the controls, and died in the usual interval (table 1, dog 3). Likewise prophylactic treatment with 70 mgm. DCA did not allow recovery when the fore treatment was followed with a transfusion of 2.5 cc. plasma protein concentrate per kgm. body weight in 2 more dogs.

It seems clear that in no case, in a series of 14 dogs subjected to a 5 hour constriction of both hind legs, did either cortical extract or DCA reduce the plasma loss into the injured tissues, hasten the repair of the leg capillaries, allow a

[5] We are indebted to Ciba Pharmaceutical Products, Inc. for generous supplies of the desoxycorticosterone acetate (Percorten) used in these experiments.

greater retention of administered plasma, increase the efficacy of a transfusion, or prevent the onset of fatal circulatory collapse.

II. *Shock following leg trauma.* When all faces of the thigh muscles of both hind legs of the etherized dog were bruised by 800–1600 rapid blows delivered with a light rawhide mallet, according to the technique of Best and Solandt (25) as modified by Gregersen and his associates (26), fatal shock followed in 33 of 37 untreated control animals (27, 28). The survival periods ranged from 2 to 8 hours, with an average of 4 hours. Shock induced by this procedure can be prevented by administering spinal anesthesia just previous to trauma, or by multiple injections of 4 per cent procaine into the skin and muscles of the area to be traumatized (27). Significant changes in hematocrit, hemoglobin, or serum protein levels do not occur in this type of shock, although the plasma volume is reduced by an average of 32 per cent.

Cortical extract (35–70 cc.) and DCA (40–70 mgm.) were given to 10 dogs subjected to muscle trauma. All animals died in shock, without significantly prolonged survival periods. Thus cortical extract and the synthetic steriod DCA were without discernible effect in preventing shock produced by trauma to muscle masses of the hind legs.

III. *Shock following venous occlusion.* The technique of Perlow and co-workers (29) was followed as closely as possible in this experiment. Using ether anesthesia, and aseptic precautions, the internal and common iliac veins of the left hind leg were exposed by retroperitoneal approach, and ligated. Twelve to 15 cc. of a freshly autoclaved, 1:20 suspension of lamp black in physiological saline was then injected into the external iliac vein, to block collateral circulation through minute vessels. It is important that the lamp black suspension be thoroughly shaken immediately before transferring to an oiled syringe, and also before injection. A ligature was placed distal to the injection site to prevent back flow of the injected suspension. The incision was then closed, ether discontinued, and the animal placed in its cage without access to water. A continuous and marked swelling of the leg followed. The blood pressure fell progressively, the pulse rate rose, and fatal shock followed in 11 of 12 control animals. The survival periods ranged from 5 to 17 hours, the average being 9 hours. The hematocrit level rose slowly but progressively from an average initial value of 50.3 per cent to 56.2 per cent, the hemoglobin level from 17.4 to 19.5 grams per cent, and the serum protein concentration from 6.05 to 6.29 grams per cent. The plasma volume, determined by the T-1824 technique (24) on 2 dogs of this series, showed a fall from an initial value of 52.5 cc. per kgm. to 25.8 cc. per kgm. body weight by the time severe shock was present. Since this reduction is much greater in degree than might be expected from the rise in hematocrit, hemoglobin and serum protein levels, it was assumed that whole blood as well as plasma was lost into the tissues of the injured leg. This conclusion was also reached by Perlow and associates on the basis of microscopic observation of the leg tissues (29).

Two more dogs were then prepared in the usual manner, but they were allowed water *ad libitum* upon recovery from the ether anesthesia. Both drank freely,

taking 500 and 550 cc. respectively, during the course of the experiment. Hemodilution followed in both cases, but there was no apparent reduction in the leg swelling, and both animals died in shock in the usual time interval.

A series of 15 more dogs was treated with DCA. Twelve of these were given dosage levels in the range reported highly effective by Katz and co-workers (14), i.e., 30–40 mgm. were given subcutaneously in divided doses at 24, 18 and 12 hours before the experiment, and 10–30 mgm. more were given intramuscularly after the operation. Six animals of the series were allowed to take water, six were not. Of those not allowed water, 5 died in shock in the usual interval. Hemoconcentration was not significantly different from that of control animals, but the serum protein levels showed a slight decline instead of the usual slight increase. All 6 animals given water drank copious amounts (the average being 1130 cc.) and, like the controls similarly treated, showed hemodilution. The serum protein levels declined from an average of 5.59 to 4.14 grams per cent. All animals died in shock, although the survival period of one was prolonged to 47 hours.

Since the possibility remained that insufficient hormone had been given, 3 more dogs were given 70 mgm. DCA as a prophylactic treatment over the 48 hours preceding the experiment, followed by an additional 40 mgm. given over the first 8 hours following the operation, to make a total of 110 mgm. per animal. Again all dogs died in shock in the usual interval. Hence in the complete series of 15 animals given DCA treatment, but one dog recovered, which, when compared with survival of the control series, indicates that DCA did not protect against the fatal shock which follows venous occlusion of the hind limb of the dog, nor was the survival period definitely prolonged.

Discussion. A study of circulatory failure in adrenalectomized dogs led to the suggestion that cortical extract might prove of value in the treatment of human shock (30). More recent study of the circulatory changes found in adrenal insufficiency, and of the factors involved in producing the extreme sensitivity of these animals to stress procedures, has thrown doubt upon the premise that this "shock-like" circulatory collapse has a similar etiology to that of shock seen in the animal with intact glands. The decreased resistance of the adrenalectomized animal to circulatory stress is apparently related to the loss of carbohydrate-active adrenal hormones, and is a manifestation of a break-down in the normal intermediary metabolism of carbohydrate in the tissues of the body (31, 32). This fundamental metabolic defect is reflected in a reduced capacity of the smooth muscles of the arterioles to maintain a prolonged contraction, and hence make sustained capacity adjustments at the vascular periphery. The capillaries likewise appear affected, becoming atonic and increasingly permeable (33). Whether this effect is also due to the lowered ability of the organism to properly metabolize carbohydrate is not yet fully established (33, 34). The heart is probably also affected (35). The ability of cortical extract and the carbohydrate active steroids to increase the resistance of the adrenalectomized animal to circulatory stress procedures, and to revive the animal from circulatory collapse, appears to depend upon the restoration of the normal metabolic

processes in the tissues concerned. So far as is known there is no evidence that a derangement of carbohydrate metabolism is concerned in the shocked intact animal whose adrenal glands are presumably functional.

The shock resulting from release of tourniquets and that following venous occlusion of the leg is due chiefly, if not entirely, to local loss of blood fluid into the limbs with resulting reduction of circulating volume. In tourniquet shock the fluid lost is chiefly plasma whereas in venous occlusion much whole blood also goes out of the circulation.

Shock following muscle trauma to the hind legs is due to two factors: 1, local fluid loss at the site of injury, and 2, nervous factors initiated by a flow of nociceptive stimuli from the traumatized area. Hence in these experiments cortical hormones appear ineffective in two types of shock, that due primarily to local fluid loss and injury of capillaries, and that due to a combination of this factor with nervous influences.

SUMMARY

1. The therapeutic value of adrenal cortical extract and of the synthetic adrenal hormone DCA, has been tested in intact dogs, shocked by three different procedures: 1, constriction of both hind legs with tourniquets; 2, trauma to muscle masses of both hind legs, and 3, venous occlusion of one hind limb. Neither extract or DCA proved beneficial in the treatment of shock resulting from these procedures.

2. An explanation is offered for the discrepancy between these negative results obtained following use of adrenal cortical extract and DCA on shocked intact dogs, and the positive results obtained with these hormones on adrenalectomized animals. This explanation is based upon the difference in etiology of circulatory failure in the two types of animals.

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THE EFFECTS OF DIRECT CHEMICAL AND ELECTRICAL STIMULATION OF THE RESPIRATORY CENTER IN THE CAT[1]

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Many attempts have been made to localize the respiratory center by progressive sectioning or destruction of the brain stem, by electrical stimulation, by study of respiratory action potentials and by clinical observations (for bibliography, see (13)). One of the most recent of these studies has been that by Pitts, Magoun and Ranson (13); they observed marked inspiratory and expiratory responses in the cat following electrical stimulation of the brain stem using the Horsley Clarke instrument, and concluded that the inspiratory and expiratory centers each occupy about 30 cu. mm. of the medullary reticular formation overlying the region of the inferior olives. This study led to a desire to obtain further knowledge concerning the actions of chemical substances introduced directly into the medulla, and to correlate results obtained from chemical and electrical stimulation at the same points.

METHODS. Cats, 2.5 to 3.5 kilos, were anesthetized with 36 mgm. of sodium pentobarbital per kilo intraperitoneally or were decerebrated under evipal anesthesia. The supraoccipital and interparietal bones were removed and the vermis of the cerebellum was scooped out thus exposing the floor of the fourth ventricle. In some cases the tentorium was removed to facilitate exploration of the middle and upper pons. The cat's head was then fixed firmly in the Horsley-Clarke stereotaxic instrument, in the electrode holder of which was fixed a fine hollow steel needle, 0.5 mm. outside diameter, insulated except at the tip. The needle was so arranged that it could be used both for injection of chemicals and for electrical stimulation. For the latter a thyratron regulated discharge was employed, the frequency of stimulation usually being 270/second and the voltage 1.5–4.0 volts measured by a cathode ray oscillograph with the needle in the medulla; a large copper rod inserted in the rectum served as the indifferent electrode.

A calibrated screw plunger was used for injecting chemicals; the amount injected varied from 1 to 5 cu. mm., and averaged 2 cu. mm. The chemicals most frequently used were sodium bicarbonate-CO_2 buffers, acids (lactic, hydrochloric, and carbonic), potassium chloride, acetylcholine, nicotine and strychnine. Blood pressure was recorded from the femoral artery by means of a mercury manometer. Respiration was measured by a spirometer to which oxygen was continuously added; CO_2 was absorbed by a cannister of soda lime between the tracheal cannula and the spirometer.

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A typical experiment was conducted as follows: first the position of the obex was carefully determined in relation to the three planes of the Horsley Clarke instrument. Then the needle, filled with the solution to be tested, was inserted into the desired point in the brain stem, the response to electrical stimulation was determined, 1 to 2 cu. mm. of the solution was injected, and the electrical response again was tried; this procedure was repeated at various spots as long as the cat remained in good condition. Since it was not feasible to locate all injection sites by serial sections in the large number of cats used (115 in all), the points have been charted in relation to the obex, the midline, and the surface of the brain stem. Since the underlying structures in the cat's brain are remarkably constant in relation to surface markings if cats of fairly uniform size are used, it was felt that the composite picture derived from a very great number of surface localizations would offset the lack of more direct and precise histological localization.

RESULTS. The effects produced by electrical responses were in all respects similar to those reported by Pitts, Magoun and Ranson. The most vigorous

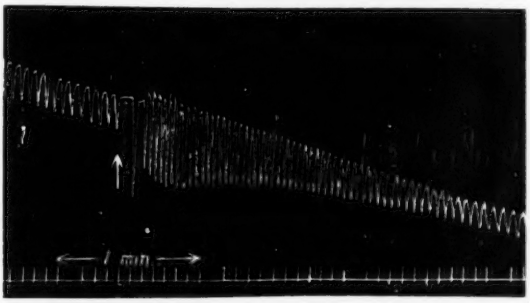

Fig. 1. Cat. Nembutal. Spirometric tracing of respiration. At the arrow, 2 cu. mm. of CO_2-bicarbonate solution (pH 7.4, pCO_2 200 mm. Hg) was injected into the medulla.

responses were elicited by stimulation in the region from 1 mm. below the obex to 3 mm. above it. Furthermore expiratory and inspiratory areas were fairly sharply defined. As a rule the former were dorsal and the latter ventral; 74 per cent of all expiratory responses were elicited by stimulation of spots 1 to 4 mm. deep, while 78 per cent of all inspiratory responses occurred following stimulation 3 to 7 mm. below the surface of the area outlined above.

While the usual effect produced by electrical stimulation was an apnea in the inspiratory, expiratory or mid position or a shifting of respiration toward inspiratory or expiratory levels, the usual effect produced by chemical stimulation was a prompt increase in depth or in both depth and rate often amounting to marked hyperpnea lasting several minutes (fig. 1). Occasionally following chemical stimuli expiratory apneas were observed and rarely inspiratory apneas were seen; in some instances the typical hyperpnea was preceded by a brief inspiratory or expiratory apnea. The effects of chemical stimuli also differed from those of electrical stimuli in that the latter repeatedly produced the same effect while the response from chemical stimulation was not fully repeatable as a rule. In a

few instances, however, three injections at the same spot led to hyperpneas of similar duration and intensity. In general the injections produced more tissue damage than the simple puncture involved in electrical stimulation. This was attested to not only by the lack of repeatability following injections, but also by the appearance of edema in histological sections and by the fact that electrical stimulation was occasionally less effective after injections than at the same spot beforehand. On the other hand as many as 80 injections totalling 230 cu. mm. of fluid were made into one cat's brain stem in addition to 90 electrical stimulations, and yet there was no outward change in respiration over a period of four hours.

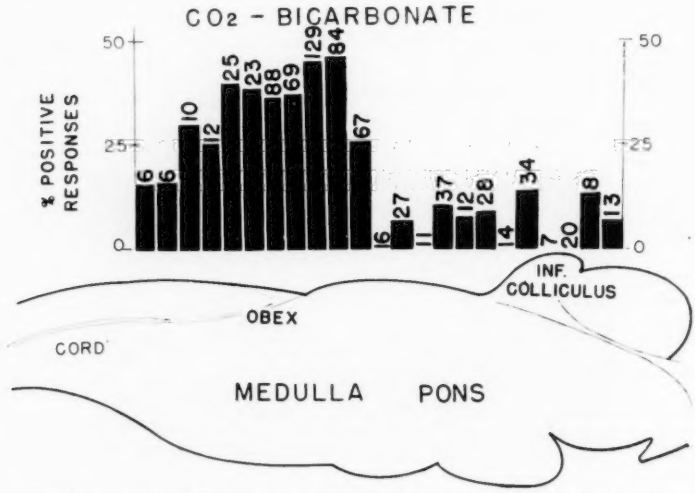

Fig. 2. Solid bars represent the per cent positive responses to CO_2-bicarbonate solutions injected into the pons, medulla and upper portion of the spinal cord. Each bar represents injections made at 1 mm. intervals into portions of the brain stem represented immediately beneath the bar. The numbers above the bars indicate the total injections performed at that level, irrespective of distance from midline or below surface of the brain stem.

The areas which yielded positive responses following chemical stimulation can be seen in figure 2. This region corresponds roughly to the respiratory center previously outlined by electrical stimulation, though the area responding to injected chemicals is slightly larger, due in all probability to a spread of the stimulants. A sufficient number of injections has not yet been made in the region of the pneumotaxic center to warrant any conclusions concerning its ability to respond to chemicals.

It is noteworthy that although the maximal effects of both electrical and chemical stimulation were obtained from the same region, at any given point close correlation was not necessarily observed. Of the spots at which chemical stimulation produced marked hyperpnea, electrical stimulation produced inspiratory

responses in 60 per cent but at 18 per cent of these points there was no response to electrical stimulation. Furthermore 14 per cent of the points which were completely negative to chemical stimulation gave inspiratory responses when stimulated electrically, and an additional 14 per cent which failed to respond to injections yielded expiratory responses following electrical stimulation (see table 1).

As shown in figure 3, one of the most effective chemical stimuli was a 1.3 per cent solution of $NaHCO_3$ buffered to pH 7.4 by CO_2 so as to have a pCO_2 of

TABLE 1

TYPE OF RESPONSE FOLLOWING INJECTION OF CO_2-$NaHCO_3$ SOLUTIONS	NO. OF OBSERVATIONS	TYPE OF RESPONSE FOLLOWING ELECTRICAL STIMULATION			
		Inspiratory	Expiratory	No change	Miscellaneous
		per cent	*per cent*	*per cent*	*per cent*
Moderate to marked hyperpnea	83	60	7	18	15
Slight hyperpnea	99	40	9	30	20
Depression of respiration—Expiratory response	80	20	60	9	11
No change	420	14	14	55	17

In this table are included only those experiments in which responses to electrical and chemical stimulation were tested at the same points.

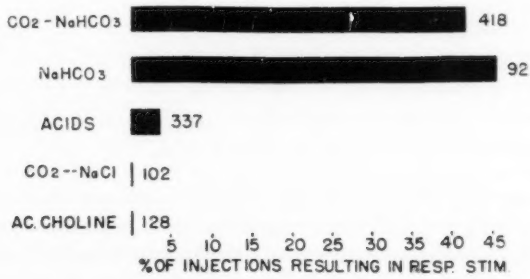

Fig. 3. Per cent of injections resulting in respiratory stimulation. This chart includes only injections into the medulla (2 mm. below to 4 mm. above the obex); however injections at all depths and at varied distances from the midline are included.

250 mm. This was effective in amounts as small as 1 cu. mm. Numerous controls such as distilled water, 0.9 per cent saline, and 2 per cent saline repeatedly gave negative results.

Of 750 injections of the CO_2-$NaHCO_3$ solution into the medulla and pons of 65 cats (from 6 mm. below to 16 mm. above the obex), 214 (29 per cent) resulted in immediate mild to vigorous stimulation of respiration (see fig. 2). Of 418 injections into the medulla alone (2 mm. below to 4 mm. above the obex) 42 per cent were positive. The latter figures include injections at all depths of the medulla and at varied distances laterally from the midline; if only the injections

in the center of this region (corresponding to the reticular formation) are considered, 80 per cent of the injections resulted in positive responses.

In addition to the responses described, injections at 80 points resulted in temporary expiratory apnea or in a decrease in rate or depth of respiration. Since in 60 per cent of the cases (table 1), these same spots responded to electrical stimulation by similar depression of breathing, it is reasonable to suppose that the CO_2-$NaHCO_3$ solution actually stimulated expiratory neurones.

It is of interest to note that a 1.3 per cent solution of $NaHCO_3$ in distilled water, without added CO_2 and having a pH of 8.0 or more, produced increase in rate and depth of breathing following 43 of 92 injections into the region of the medulla. Other bicarbonate solutions, hypotonic and hypertonic, with a pCO_2 of 100 to 300 mm. Hg and a pH of 7.4 produced similar responses. No consistent changes in blood pressure were observed.

In striking contrast to these effects, acid solutions (N/1000, N/100, N/10 hydrochloric or lactic acid) when injected 337 times in amounts of 1 to 5 cu.

TABLE 2

TYPE OF RESPONSE FOLLOWING INJECTION OF ACID SOLUTIONS	NO. OF OBSERVATIONS	TYPE OF RESPONSE FOLLOWING ELECTRICAL STIMULATION			
		Inspiratory	Expiratory	No change	Miscellaneous
		per cent	*per cent*	*per cent*	*per cent*
Moderate to marked hyperpnea	2	100			
Slight hyperpnea	12	32	16	24	24
Depression of respiration—Expiratory response	78	28	28	22	22
No change	254	40	21	19	20

In this table are included only those experiments in which responses to electrical and chemical stimulation were tested at the same points.

mm. into the areas of the medulla shown to be most sensitive to CO_2-$NaHCO_3$ solutions, produced only minimal responses: in 8 instances barely perceptible stimulation of respiration occurred, in 4 slight hyperpnea occurred and in one, an inspiratory apnea developed. Thus acid solutions produced only 3.8 per cent positive responses compared with 42 per cent positive responses following CO_2-$NaHCO_3$ solutions injected into similar areas (see fig. 3). Furthermore in many instances, following failure of lactic acid to stimulate, injection of a CO_2-$NaHCO_3$ solution into the same spot led to definite respiratory stimulation. In addition, acids often showed a definite tendency to depress respiration even in regions in which electrical stimulation did not produce similar responses. In 78 instances, acid injections led to expiratory apnea or to a decrease in rate or depth of breathing, but at only 28 per cent of these spots did expiratory responses follow electrical stimulation and at an equal number (28 per cent) inspiratory responses followed electrical stimulation (see table 2). This differed markedly from the expiratory responses produced by injections of CO_2-$NaHCO_3$ solutions (see above).

Other acid solutions likewise failed to stimulate: saline solutions equilibrated with CO_2 at 70, 300, and 700 mm. pressure (pH 3.9–5.0) were injected 102 times with no respiratory responses.

Other substances injected were: 1.2 per cent KCl which stimulated in 12 of 57 injections (21 per cent) but frequently led to depression either initially or following transient stimulation; 1 to 5 per cent nicotine which stimulated weakly in 4 of 45 injections (9 per cent); 1 per cent strychnine which produced 2 doubtfully positive responses in 87 injections; 0.01 to 1 per cent acetylcholine, which in 128 injections, produced no clear cut instance of respiratory stimulation. Sodium cyanide, metrazol, sodium citrate, aminophylline, ether, sodium carbonate and carbonic anhydrase were injected in a few experiments, all with negative results, but none of these was investigated sufficiently to permit definite conclusions to be drawn.

Discussion. Haldane's original concept of the specificity of CO_2 as the main physiological regulator of respiration has been modified several times since it was first suggested in 1908. The most widely accepted modification considers that respiration is controlled not necessarily by the level of arterial pCO_2 or even by arterial pH but by the internal acidity of the cells of the respiratory center (5). This latter hypothesis was necessary at the time to provide an explanation for the numerous instances in which respiration increased in the face of a decrease in arterial pCO_2 or of an increase in arterial pH; it suggested that though the arterial blood may become more alkaline during hemorrhage, anoxemia, etc., the cells of the respiratory center may actually become more acid and so provide the stimulus for the hyperpnea occurring under these conditions. Since 1925 however, the need for this conception has been progressively diminished by important physiological discoveries which have established beyond doubt the existence of respiratory reflexes hitherto unknown. It is now known for example that stimulation of respiration may arise reflexly from the pressure receptors in the aortic arch and carotid sinuses (due to a fall in arterial pressure as in hemorrhage) or from the chemoreceptors of the carotid and aortic bodies (due to a decrease in arterial pO_2 as in anoxemia) (9) or from proprioceptors of the muscles, tendons and joints (as in muscular exercise) (8, 2). Thus the important exceptions to Haldane's concept of the specificity of CO_2 can now be satisfactorily explained by the effects of these new reflexes and the need for conjecture no longer exists. Indeed Gesell, who formerly championed the intracellular acidity concept, recently has reversed his previous position by accepting arterial pH values as indicative of changes in pH within the cells of the respiratory center (6). Evidence against the "acid" hypothesis has been presented in this paper since bicarbonate-CO_2 buffers usually stimulated the cells of the respiratory center while acid solutions rarely stimulated and often depressed the center.

The observation that sodium bicarbonate solutions with a pH of 8.0 can stimulate respiration as vigorously as bicarbonate-CO_2 mixtures is not surprising in view of the work of Jacobs (11) who found that the CO_2 in bicarbonate solutions penetrates into the interior of cells much more rapidly than the HCO_3 ion.

However the failure of CO_2-saline solutions to stimulate in the present experiments is surprising. One explanation might be that such solutions were so acid that any tendency to stimulate by reason of CO_2 excess was offset by a tendency to depress by reason of excess acidity. Another explanation might be that high tensions of CO_2 exerted a narcotic action, but in this case transient stimulation might be expected to precede the depressant effect; furthermore CO_2-saline solutions with a pCO_2 of only 70 mm. failed to stimulate. It is probable that no simple explanation will suffice and that the situation is a complex one. It is interesting to note that the most favorable condition for stimulation of respiration by means of localized injections appears to be one in which the CO_2 molecule may penetrate to the interior of the cell while the exterior is bathed in a bicarbonate solution (i.e., CO_2-bicarbonate buffers or bicarbonate solutions).

Since all of the concepts of respiratory control that have been proposed by Haldane, Hasselbach, Winterstein and Gesell have specified either the CO_2 molecule or acidity as the specific stimulus to the respiratory center, it has become almost obligatory to interpret all new evidence in terms of CO_2 or acidity. However figure 3 shows quite clearly that the presence of bicarbonate appears to be necessary for stimulation, while the presence of a high CO_2 tension or of acidity without bicarbonate does not seem to be necessary. Therefore the possibility exists that the HCO_3 ion may be a very important factor in the control of respiration as suggested previously (1, 4). The present experiments cannot be considered as conclusive evidence because of the unusual technique employed: cerebellum, and occasionally cerebrum, removed; insertion of needle into medulla; injection of fluid tending to produce edema and lack of repeatability; sudden gush of stimulant material directly in contact with medullary cells, etc. However in favor of the validity of the results obtained by these unorthodox methods are the following facts: the responses often resembled the hyperpnea produced by CO_2 inhalation; they occurred in the region of the respiratory center as outlined by electrical stimulation; bicarbonate solutions produced a high percentage of positive responses, whereas acids (including carbonic acid) rarely produced hyperpnea; and finally bicarbonate solutions occasionally stimulated respiration when injected at points which had failed to respond to injection of acid a moment before.

The effects produced by injection of KCl (transient stimulation followed by depression or initial depression) are similar to those previously obtained upon respiration by perfusion of the brain (10) and of the carotid bodies; the same effects have also been observed following direct application of potassium to nerve cells (3, 12).

In view of the recently proposed neurohumoral concept of respiratory control (6), special attention should be drawn to the fact that acetylcholine failed to stimulate though injected 128 times directly into the regions of the respiratory center previously shown to be most sensitive to CO_2-bicarbonate buffers. Occa-

sional delayed hyperpnea was observed when high concentrations of acetylcholine were injected, but these can be attributed entirely to reflexes aroused by a fall in blood pressure since the hyperpnea did not occur following carotid sinus and aortic arch denervation or when the fall in blood pressure was prevented by atropine. Attention should also be called to the point that injection of chemicals into the fourth ventricle (7) is not equivalent to localized injections into the medulla. It is probable that systemic absorption takes place more readily than penetration through the 5 mm. of nervous tissue that separate the respiratory center from the surface of the brain stem. Nicotine, for example, often produced respiratory stimulation when dropped on the floor of fourth ventricle but this effect was usually abolished by denervation of the carotid and aortic bodies.

A final point which merits comment is the different types of respiratory response elicited by chemical and by electrical stimulation: chemical stimulation rarely produced an inspiratory apnea while electrical stimulation often produced intense and prolonged apneas; chemical stimulation usually led to increase in both rate and depth of breathing while electrical stimulation rarely did. It is doubtful if this can be explained upon the basis of different frequency or intensity of stimulation in the two cases, for varying the frequency and strength of the electrical stimulus over wide ranges failed to convert the apnea to a hyperpnea. It is conceivable that the electrical stimulus affected predominantly groups of axones all of which originated from inspiratory or expiratory areas, while the chemical stimulus may have affected only cell bodies. Another possible explanation is that the chemical substances spread along the needle tract and so reached both expiratory and inspiratory neurones simultaneously, whereas the electrical stimuli were more sharply localized. The explanation that seems most attractive is that the electrical stimulus only duplicates the result of neuronal activity while chemical stimuli act through one normal cause of that activity, i.e., by eliciting changes in chemical equilibria within the neurons of the center.

SUMMARY

The effects of direct chemical and electrical stimulation of the brain stem were compared in a series of 115 cats using a modification of the Horsley Clarke stereotaxic instrument. Injections of minute amounts (2 cu. mm.) of CO_2-bicarbonate mixtures were followed often by immediate hyperpnea, particularly when these were injected into the region of the respiratory center previously outlined by Pitts, Magoun and Ranson. Acids (carbonic, lactic, hydrochloric), injected in the same manner, rarely stimulated respiration and often led to respiratory depression. The significance of these findings in relation to the current concepts of respiratory control is discussed. The response to the injection of a number of other chemicals (sodium bicarbonate, hypertonic and hypotonic sodium chloride solutions, nicotine, strychnine and acetylcholine) was also tested.

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THE EFFECT OF 17-HYDROXY-11-DEHYDROCORTICOSTERONE ON THE GROWTH OF YOUNG ADRENALECTOMIZED RATS

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One of the most convenient methods for the assay of the adrenal steroids and whole extracts is that employing the young adrenalectomized rat. In a previous communication from this laboratory (1) describing this method of assay, it was pointed out that the growth of the young adrenalectomized rats is not proportional to dosage and that "growth alone is not a satisfactory basis of assay." However, there can always be determined a certain minimum dose which, administered by single subcutaneous injection daily for 20 days to the 4-weeks-old adrenalectomized male rats weighing 50 to 60 grams, is sufficient to maintain life in at least 80 per cent of the rats and produce an average growth of at least 20 grams for the 20 day period. In this test the emphasis is upon survival, and the growth requirement only insures that the treated animals gain more weight than similar animals without treatment. This criterion of survival and growth of adrenalectomized rats as a measure of hormonal activity of whole extracts has been questioned.

Ingle (2), studying the weight changes for a period of 7 days in rats having an initial weight of 100 grams, observed that adrenalectomized rats treated with 11-desoxycorticosterone acetate and rats having sham operations, made substantial gains in weight during this period, whereas adrenalectomized rats treated with similar quantities of 17-hydroxy-11-dehydrocorticosterone lost weight. When normal rats of 180 grams or above were treated with massive doses of 17-hydroxy-11-dehydrocorticosterone, inhibition of growth and a definite loss of body weight were observed (3). Wells and Kendall (4) reported an inhibition of growth in young normal rats treated with 1.0 mgm. of 17-hydroxy-11-dehydrocorticosterone per day for 10 days. On the basis of these observations, Kendall has expressed the opinion (5) that the presence of this "growth-inhibiting" steroid in adrenal cortex extracts would mask the effect of other growth-promoting principles present in extracts and thus make the survival-growth test invalid as an assay method. No studies under the experimental conditions of this assay method have been reported by Kendall. Also of interest in this respect are the observations by Ingle (2) that when 17-hydroxy-11-dehydrocorticosterone was mixed with 11-desoxycorticosterone the rats gained weight and there was no tendency toward inhibition of growth until massive dosages were administered.

In an earlier publication from this laboratory (6), it was reported that, at a dosage level of 1.0 mgm. per rat per day, 17-hydroxy-11-dehydrocorticosterone and 17-hydroxy-corticosterone maintained the life and supported growth of 4-weeks-old adrenalectomized rats of the Wistar strain. Complete assays were

not run at that time because of the small quantity of pure steroids at hand. Since more of the pure substances have now been obtained, we have been able to investigate more completely the effect on the growth of the young rats. The results of these studies demonstrate that 17-hydroxy-11-dehydrocorticosterone will maintain the young 4-weeks-old adrenalectomized rats and support growth at dosage levels of $\frac{1}{8}$ to 1 mgm. per day.

EXPERIMENTAL. Two separate experiments were carried out in this study. In each experiment, male rats of the Sprague-Dawley strain weighing 50 to 60 grams were adrenalectomized at 28 days of age. The diet was Purina Dog Chow. The animals were weighed immediately before adrenalectomy and weights were then recorded at the same hour each day until the death of the animal. The first daily injection was administered after the operation, and the same dose was repeated for the next 19 days making twenty doses in all. The materials were administered in sesame oil by subcutaneous injection and the concentrations were adjusted so that the volume of oil injected was between 0.1 and 0.2 cc. All of the rats were housed in air-conditioned animal rooms which had a temperature range of 75–79°C.

In experiment 1, the 17-hydroxy-11-dehydrocorticosterone was obtained in this laboratory by crystallization from beef adrenal gland concentrates. Its identity was proved by melting point, specific rotation, analysis, crystalline form, and comparison with a known standard in the muscle contraction test (7). The rats were adrenalectomized by the procedure previously described (1). The test substance was administered at the five dosage levels of 0.25, 0.125, 0.1, 0.067, and 0.05 mgm. per rat per day. For purposes of comparison similar dosages of a sesame oil solution of whole adrenal cortex extract were also administered.

Experiment 2 was carried out by one of us (D. J. I.) independently. This was an attempt to duplicate for the 4-weeks-old rats, weighing 50–60 grams, the experimental conditions used in the previously reported study (2) on 180 gram rats. The sample of 17-hydroxy-11-dehydrocorticosterone used in this experiment was prepared by Dr. J. J. Pfiffner of Parke, Davis and Company, Detroit, Michigan and had been found by one of us (D. J. I.), in studies conducted at the University of Pennsylvania, to be ineffective for maintaining the growth of adrenalectomized male rats of 180 grams weight. Bilateral adrenalectomies were performed under ether anesthesia according to the method of Ingle and Griffith (8). Dosage levels of 1.0, 0.25 and 0.1 mgm. per rat per day were studied and the weight changes were compared to those of sham-operated rats and untreated adrenalectomized rats.

RESULTS. The results obtained by the administration of the test substances in experiment 1 are summarized in table 1. The results obtained by the administration of 17-hydroxy-11-dehydrocorticosterone in experiment 2 are summarized in figure 1. From the results of each of the two experiments it is apparent that at dosages of $\frac{1}{8}$ to 1 mgm. per rat per day, 17-hydroxy-11-dehydrocorticosterone maintained life in these adrenalectomized rats over the 20 day period of injection and permitted about the same amount of growth as effective dosages of adrenal cortex extract. The rate of growth was not as great, how-

ever, as that of the non-adrenalectomized control rats (fig. 1) which were subjected to false operations. The greatest growth response was elicited by the highest dose (1.0 mgm.) of the pure compound.

DISCUSSION. The results clearly indicate that 17-hydroxy-11-dehydrocorticosterone is capable of supporting life and growth in 4-weeks-old adrenalectomized rats under the experimental conditions of the assay test, even when amounts are administered which are greater than those occurring in physiologic doses of cortical extracts. We have also carried out preliminary experiments with 17-hydroxycorticosterone which show that this compound will also maintain life and support growth under the conditions of the assay test and possesses, likewise, an activity of 8 to 10 rat units per milligram. These effects are char-

TABLE 1

The effect of 17-hydroxy-11-dehydrocorticosterone on the growth and survival of four-weeks-old male rats

SUBSTANCE	DAILY DOSE	NUMBER OF RATS INJECTED	AVERAGE SURVIVAL	AVERAGE GROWTH	NUMBER OF RATS SURVIVING 20 DAYS	AVERAGE GROWTH OF SURVIVING RATS ON 21ST DAY	AVERAGE SURVIVAL AFTER LAST INJECTION
			days	*grams*			
17-hydroxy-11-dehydrocorticosterone	0.25 mgm.	10	23.5	30	9	31	4
	0.125 mgm.	5	22	22	5	22	2
	0.10 mgm.	9	24.5	18.5	9	18.5	4
	0.067 mgm.	13	24.0	16.0	8	21.0	8
	0.05 mgm.	13	17	12.0	2	18.0	8
Oil solution. Adrenal cortex. Extract 105–DBK–2. 1 cc. 80 gm. gland	0.2 cc. 16 gm.	10	26	27	10	27	6.0
	0.15 cc. 12 gm.	4	24	28	4	28	4.0
	0.10 cc. 8 gm.	15	26	32	12	38	8.0
	0.075 cc. 6 gm.	10	21	13	6	18	4.0
	0.05 cc. 4 gm.	9	23	23	6	30	6.0
Sesame oil controls	0.2 cc.	27	7.2	3.4	0	—	—

acteristic of active adrenal cortex extracts administered in physiologic amounts and of the normal secretion of the animals' own adrenal glands which is assumed to include 17-hydroxy-11-dehydrocorticosterone. Our results, therefore, offer evidence supporting the validity of the survival-growth method of assay as previously described (1).

Adrenal cortex extracts and pure adrenal cortical steroids influence both electrolyte and carbohydrate metabolism (9, 10). This has stimulated a great amount of study to develop tests for evaluating the physiologic activity of extracts and pure compounds with respect to these specific functions. No one of these tests, however, should be considered adequate in itself as an assay procedure for measuring the biologic activity of whole adrenal cortex extracts,

however important it might be for following the activity with respect to one function. If either carbohydrate function tests or electrolyte balance studies are used exclusively, important fractions of the extract may be overlooked. The survival-growth test is responsive to the composite activity of adrenal cortex extracts both with respect to the known compounds and the amorphous fractions. We know of no adrenal steroid fraction which has been found active by other valid methods of assay which is not active by this test. It should, indeed, be pointed out that prolongation of the survival of adrenalectomized animals and of patients having adrenal cortical insufficiency is the most important objective to be attained in the preparation of whole adrenal cortex extracts. A biological test of this property should then logically be based upon a prolongation of survival time.

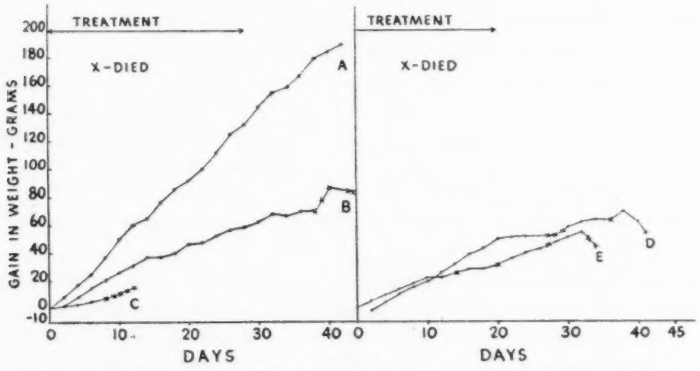

Fig. 1. Weight changes of 28 day old male, adrenalectomized rats treated with 17-hydroxy-11-dehydrocorticosterone.

A, sham operated; *B*, 0.25 mgm. daily; *C*, sesame oil only; *D*, 1.0 mgm. daily; *E*, 0.1 mgm. daily.

The reason for the discrepancy in the results obtained earlier by one of us (D. J. I.), using rats of 180 grams weight, and the results of the present experiments, using rats of 50 to 60 grams weight, is not understood. We have insufficient data on simultaneous comparisons of the two weight groups and shall not draw conclusions at this time.

SUMMARY

The effect of 17-hydroxy-11-dehydrocorticosterone, at daily dosage levels of 0.05 to 1.0 mgm., on the survival and growth of 4-weeks-old adrenalectomized rats was determined. It was found that the compound at doses of one-eighth to one milligram is capable of maintaining the rats and supporting their growth. Whole adrenal cortex extracts which contain 17-hydroxy-11-dehydrocorticosterone and similar steroids can therefore be assayed for their complete cortical hormone activity by the method as previously described (1).

Acknowledgment. We wish to express our appreciation to Dr. J. J. Pfiffner of Parke, Davis and Company who supplied the sample of 17-hydroxy-11-dehydrocorticosterone used in experiment 2.

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BACK-DIFFUSION OF UREA IN THE MAMMALIAN KIDNEY

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As recognized in 1921 by Austin, Stillman and Van Slyke (1), the rate of urea excretion is dependent on the volume flow of urine. Rehberg (11) pointed out that if the clearance of creatinine in man be taken as a measure of glomerular filtration, it would follow that urea, presumably filtered in concentration equal to that of the plasma, must be partly reabsorbed from the tubular urine. He suggested that urea might escape by diffusion from the concentrated tubular urine. Although from later work it appears that the creatinine clearance in man (but not in the dog) overestimates filtration rate, owing to some tubular secretion, Rehberg's inference of urea reabsorption has been confirmed by the use of excretable substances which give more probable estimates of filtration rate: ferrocyanide in the dog (14), creatinine in the dog (12), inulin in dog and man (13). All these studies indicate that about 40 per cent of the filtered urea is reabsorbed under conditions of moderate or high urine flows and a greater amount with low flows. Shannon pointed out, however, that a single diffusion process could not explain the phenomenon, since such a process if capable of causing the 40 per cent loss of filtered urea at high flows, would at moderately low flows lead to practical equilibration of urine and blood, contrary to experience. Hence he suggested that a minimum of two independent diffusion processes might operate, located in the proximal and distal parts of the tubules, and accompanying respectively the fixed "obligatory" reabsorption of about 90 per cent of the filtered water and the "facultative" reabsorption of variable proportions of the remaining 10 per cent of the water. In this connection "obligatory" and "facultative" are used as applied by Homer Smith (13). These conclusions, derived from the study of dogs, were extended to human kidneys by Chasis and Smith (2), who also obtained data on urea reabsorption in cases of chronic nephritis.

The equations of Austin, Stillman, and Van Slyke (1) relating urea clearance to urine volume over volume ranges above 0.5 cc. per minute, the equation of Chesley (4) for lower volume ranges, and the more comprehensive equation of Dominguez (5) covering all volume ranges, all give fairly good fits with observed data; but they are all purely empirical formulations. None of them is derived from known physico-chemical laws.

The formulation presented in this paper is based on the assumption that the decrease in urea clearance with urine volume is caused by a tubular reabsorption of urea in accordance with the quantitative laws of diffusion. The comprehensive equation thus deduced is found to fit observed data. Such fit does not, of course, prove the validity of the assumptions on which the equation is based. The simple hypothesis doubtless oversimplifies actual conditions, but it has the

advantage that it leads to a workable equation, consistent in its assumptions with the facts of renal excretion in so far as they are at present known, and capable of quantitative test by further observations.

The present formulation is offered for what assistance it may give in clarifying the steps of the excretory process. It is not recommended for clinical use as a substitute for the "maximal" and "standard clearance" formulae (9), which are simpler, and are adequate for their purposes, except for conditions of unusually small urine flows (4, 8).

Simplifying assumptions for the present analysis are:

a. Concentration of tubular urine is uniform in any cross-section of a tubule (i.e., axial flow and turbulence effects are neglected).

b. Tubule volume and permeability of the wall to urea are constant under varying conditions of flow.

c. Water reabsorption occurs at a uniform rate per unit length of tubule in each of the anatomical portions which correspond to the two assumed phases of water reabsorption, but this rate is different in the two portions.

d. The resistance to urea diffusion through the tubular wall will be treated as if it were concentrated in a single thin membrane. Actually, of course, urea returning from tubules to blood vessels must pass through tubular cells, cell boundaries and tissue space.

e. Behavior of individual tubules is sufficiently uniform that the performance of the kidney as a whole may be predicted from the analysis of a single tubule.

f. The influence of blood level on urea concentration gradient will be neglected since it is usually comparatively low and constant; when this factor is not neglected it leads to a more complicated expression differing by less than 2 per cent in predicted values.

Consider a section of tubule of radius, r and length, l_1 (fig. 1). Any arbitrary cross-section of it may be identified by the distance, l, from the origin. The volume flow of urine in cubic centimeter per minute across the plane of this cross-section will be designated by v. When the urea concentration, u at this plane is known, the rate of passage of urea along the tubule across this plane may be calculated as the product, uv. The rate, uv, at which urea crosses any plane would be the same throughout the tubule, regardless of water reabsorption, if no urea were reabsorbed; u and v would vary inversely, and the product would be constant. However, when urea is reabsorbed uv must decrease as the urine passes away from the origin. It is desired to know the rate at which urea leaves the section of tubule, given the rate, u_0v_0, at which it enters the section, the rate, R, at which water is being reabsorbed per unit length of tubule and the permeability, h, of the wall to urea.

From Fick's law it is known that the rate of diffusion across a membrane is given by the product of area, permeability coefficient and concentration gradient. Expressed as a differential equation this becomes:

$$\frac{d(uv)}{dl} = -(2\pi r)hu$$

Also we have assumed that water is reabsorbed at a uniform rate per unit of tubular length, so that the volume flow across this arbitrary plane will be less than the flow at entry by the volume flow absorbed, which is $R \times l$.

$$v = v_0 - Rl$$

Eliminating l between these two equations, one obtains:

$$\frac{d(uv)}{uv} = \frac{2\pi rh}{R}\frac{dv}{v}$$

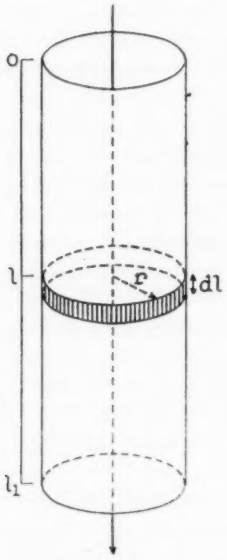

Fig. 1

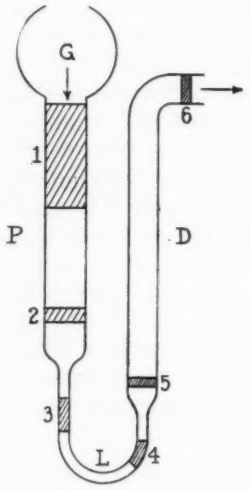

Fig. 2

Fig. 1. Arbitrary uniform section of tubule.

Fig. 2. Diagram of water absorption in a single nephron (adapted from Smith (13)). A unit portion of filtrate leaving glomerulus, G, has been reduced to about one-tenth its original volume in passage through the proximal tubule, P, but with little increase in concentration of dissolved materials. In the loop, L, a further variable reabsorption of water occurs with concentration of the solutes. Volume remains constant in distal portion and subsequent course to the bladder.

Integrating:

$$\ln \frac{uv}{u_0v_0} = \frac{2\pi rh}{R} \ln \frac{v}{v_0} = \frac{2\pi rhl}{Rl} \ln \frac{v}{v_0}$$

Let $q = 2\pi rl$ designate the total internal area of tubular wall of the section from entry to exit, and nq the area of corresponding sections in n nephrons. Also, for simplicity, let k represent the product nqh. Then the rate at which urea emerges is given by:

$$u_1v_1 = u_0v_0e^{\frac{-nqh}{v_1}\left(\frac{\log v_0/v_1}{v_0/v_1-1}\right)} = u_0v_0e^{-\frac{k}{v_1}\left(\frac{\log v_0/v_1}{v_0/v_1-1}\right)} \tag{1}$$

When no water is reabsorbed, $v_0 = v_1$. The factor $\frac{\log v_0/v_1}{v_0/v_1 - 1}$ becomes unity in this case as may be seen by setting $v_0/v_1 - 1$ equal to x, expanding $\frac{\log (x + 1)}{x}$ in a power series and letting x approach zero.

Thus when no water reabsorption occurs, the expression reduces to:

$$u_1 v = u_0 v e^{-\frac{k}{v}} \tag{2}$$

Trial of these two equations indicates that the best fit of the data is obtained by a combination of the two, which is equivalent to assuming that urea reabsorption occurs successively in two phases, one associated with a concurrent water reabsorption and the second in a portion of tubule from which no water is reabsorbed. It seems reasonable to associate the first with the 90 per cent water reabsorption in the proximal tubule, and to place the second phase of urea absorption distal to the second phase of water reabsorption (fig. 2). It will be noticed that no allowance is made for urea reabsorption concurrent with the second phase of water reabsorption, since if any significant part of the urea loss is assumed to occur there, the equation fits the data less well. It seems improbable that there is no loss of urea from the tubules concurrent with the second phase of water reabsorption; it might be, however, sufficiently small to be neglected in a first approximation. It is therefore assumed that with respect to the fate of water and urea in the tubules a physiological division may be made into three successive phases. In the first both substances are reabsorbed, in the second water alone, and in the third urea alone. Tentatively, these may perhaps be identified with proximal tubule, loop and remainder of the path including the distal tubules and collecting ducts.

Multiplying equations (1) and (2) one obtains an equation which relates plasma urea clearance $Cl_u = \frac{uv}{p}$ to filtration rate (F):

$$Cl_u = F \times e^{-\left[\frac{k_1}{v'}\left(\frac{\log F/v'}{F/v'-1}\right) + \frac{k_2}{v}\right]} \tag{3}$$

All variables in this equation are subject to measurement except v' which represents the volume flow of urine in the tubules at a stage between the first and second phases of water reabsorption, perhaps at the junction of the proximal tubule and the loop. Furtunately, it appears that v' is relatively constant under usual conditions in normal subjects, so that when filtration is approximately constant the first term of the exponent may be grouped into a single constant for purposes of testing the equation. Then:

$$Cl_u = F \times \phi \times e^{-\frac{k_2}{v}}$$

This factor, ϕ, which represents the fraction of filtered urea escaping reabsorption in the proximal tubules, appears to have a value of about 0.60, since 40 per cent of filtered urea appears to be absorbed in the proximal tubules. The product $F \times \phi$ gives the "maximal urea clearance" toward which observed urea clearance

appears to rise when urine flow is above the "augmentation limit" of Austin, Stillman and Van Slyke (1).

In order that the constant, k_2 may be determined from available data it is convenient to plot logarithmically the ratio $\frac{Cl_u}{F}$ against reciprocal of urine volume flow. This would theoretically yield a straight line since:

$$ln \frac{Cl_u}{F} = ln\phi - k_2\left(\frac{1}{v}\right)$$

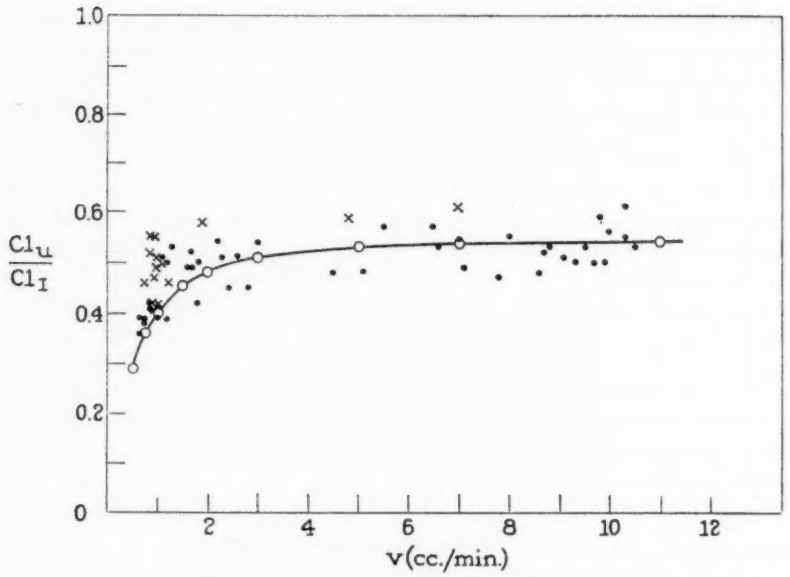

Fig. 3. Fit of theoretical equation to data from one normal human (subject T. G. (2)). Cl_u and Cl_i are plasma clearances of urea and inulin respectively. v is volume flow of urine, uncorrected for surface area. • Observed ratios on falling flows. × Observed ratios on rising flows (disregard in judging fit of the curve). ○ Theoretical points from equation $\frac{Cl_u}{Cl_i} = 0.57e^{-\frac{.36}{v}}$.

Aberrant clearance values due to clearance increases on rising urine flows (12) must be excluded since the simple conditions of a steady state, postulated in this analysis, are not maintained in such transitional states. Moreover, it is implied in the above simplified equation that constant filtration and constant reabsorption in the proximal tubule provide a constant v'. In extreme diuresis it appears that proximal reabsorption diminishes. This diminution appears to begin when urine flow exceeds about 10 per cent of filtration rate in normal humans (2) and about 5 per cent of filtration rate in normal dogs (12).

Normal human kidneys. A fit of the theoretical equation to data from one normal human (subject T. G. (2)) is shown in figure 3 which is a plot of the ratio

$\frac{\text{urea clearance}}{\text{inulin clearance}}$ against volume flow of urine. Points identified by crosses are data observed on rising urine flows; these are shown in the plot to illustrate the necessity of excluding such values.

Data covering very low urine flows (below 0.5 cc. per min.) are not included in the above paper, but are to be found elsewhere. French workers (3) over a decade ago while studying the oliguria associated with hypochloremia noted that the urine-blood ratios of urea concentration were low at extremely low flows. Chesley (4) has noted this phenomenon in oliguric normal women and found that the "standard clearance" square root correction (9) fails to make a satisfactory adjustment of what apparently is the normal behavior of urea clearance in the

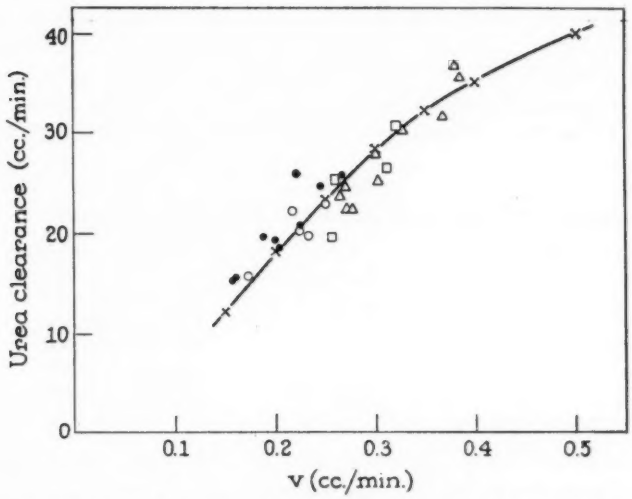

Fig. 4. Fit of theoretical equation to data from four normal oliguric subjects (4). Volume flows corrected for surface area and data from each subject multiplied by a factor to bring them on the same curve as in the original paper. • A, ○ B, △ C, □ D. × Theoretical values from equation $Cl_u = 68e^{-\frac{.26}{v}}$.

flow range below 0.35 cc. of urine per minute. Figure 4 shows a plot of data taken from his paper (4) with the theoretical curve of the present paper fitted to it. It is to be noted that the constants (68 and 0.26)[1] have approximately the same magnitude as those in the equation fitting the data at higher flows (fig. 3). Similarly the extreme depressions of urea clearance observed by McCance in the oligurias of salt deficiency and of diabetic acidosis (8) may be explained.

[1] If filtration at a normal rate of 120 cc/min be assumed, the ratio $\frac{\text{maximal clearance}}{\text{filtration}} = \frac{68}{120} = 0.57$. The corresponding ratio in the equation of figure 3 is also 0.57.

Dominguez (5) recognized that there must be some limit to the urinary concentration of urea but obtained it by extrapolating his equation to zero flows. As volume flow approaches zero, however, the time available for diffusion becomes indefinitely prolonged; it would seem more reasonable to expect equilibration between urine and blood at this limit unless active secretion of urea by the tubules is assumed. From the diffusion equation developed in this paper the maximum urine urea concentration is to be expected at volume flows somewhere between 0.15 and 0.30 cc. per minute, a limit that cannot be accurately fixed because of uncertainty of knowledge as to the behavior of glomerular filtration in this range. Owing to the technical difficulties of obtaining extremely low urine flows, and of measuring accurately these flows and the filtration rate, it is not at present possible to make a crucial test of the prediction that the concentration ceiling of urea will be reached before urine flow is stopped and that with further decline of flow, urea will appear in not greater but lower concentration.

Normal dog kidneys. A quantitative difference in reabsorption by human and dog kidneys is to be anticipated since the surface area available for back diffusion is much less in the dog. This follows not only because the individual nephron is

TABLE 1

Number of nephrons per kidney in different species, estimated by glomerular counts (10)

SPECIES	NUMBER OF GLOMERULI PER KIDNEY
Man	700,000–1,200,000
Dog	400,000– 500,000
Cat	150,000– 225,000
Rat	20,000– 30,000
Rabbit	90,000– 250,000

somewhat smaller but more importantly because of the much smaller number of nephrons, as demonstrated by the technique of glomerular counts (table 1). The variation of number of nephrons from individual to individual is of interest since it offers one explanation of the variations in urea reabsorption.

If the permeability of the dog tubules is of the same magnitude as of human tubules, the exponent of the diffusion equation should be about one-fifth to one-tenth that found from human data. This is found to be the case, as illustrated in figure 5 taken from Shannon's data (12).

So far only that part of the equation relating to distal reabsorption has been discussed. If one assumes that at flows above those yielding a creatinine U/P ratio of about 20 (corresponding to a volume flow of about 2–4 cc. per min. in a dog) the distal reabsorption in the dog is essentially abolished, then volume flow may be taken as an index of the flow emerging from the proximal tubule. With this value, which corresponds to the quantity v' in the more general diffusion equation, it is possible to make a test against the data observed during forced diuresis in the dog (12). It may be said that theory accords with experiment in predicting an increase of $\frac{\text{urea clearance}}{\text{filtration}}$ ratio that approaches the limit of unity

as volume flow approaches the filtration rate. This cannot be taken as very strong support for the theory, however, since the curve followed by this increasing ratio is not accurately defined nor the assumption set forth above adequately supported.

Abnormal human kidneys. From examination of the diffusion equation (equation 3) it may be seen that three chief factors separately or in combination may be expected to alter the relation of urea clearance to volume flow in subjects with renal disease:

a. Filtration may be reduced, with the consequence that volume flows of urine which would be only moderate in a normal kidney become equivalent to marked diureses. A flow of 5 cc. per minute would, for instance, be only 4 per cent of the filtration rate if filtration were normal at 125 cc. per minute but would be 20 per cent if filtration were reduced to 25 cc. per minute.

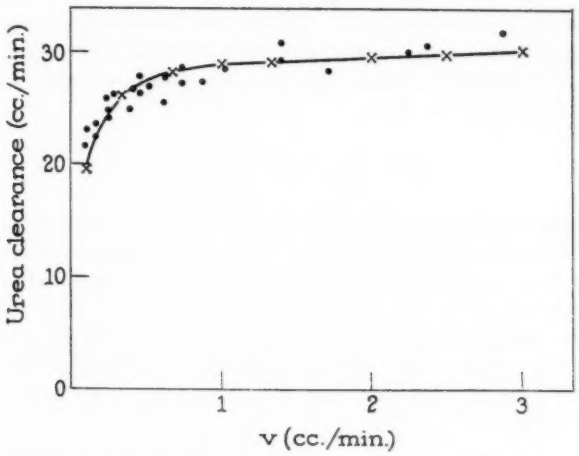

Fig. 5. Fit of theoretical equation to data from one normal dog (dog E, (12)). • Observed values. × Theoretical values from equation $Cl_u = 30e^{\frac{-.042}{v}}$.

b. Tubular surface area may be reduced so that the reabsorption observed in normal kidneys at low flows would be evident only at proportionately lower flows.

c. The capacity for facultative reabsorption of water may be reduced, thus preventing the attainment of sufficiently low flows to allow appreciable reabsorption in the distal part of the tubules. In this case variations in urine flow must be largely due to variations in filtration or in proximal tubular water reabsorption or in both, so that the percentage reabsorption of filtered urea becomes dependent not only on volume flow of urine but also on the values of these two additional variables.

Sufficient data are not at present available to permit satisfactory test of this theory. The data of Chasis and Smith (2) on the relation of urea clearance to urine flow and filtration rate in nephritic subjects show general conformity to

the theoretical curves but are insufficient for present purposes. The data reported by Hayman (6) on atypical clearances following relief of prostatic obstruction show a continuous rise of clearance with volume flow. This relation would likewise be predicted if it is assumed that the tubular capacity for facultative water reabsorption has been impaired by damage due to back pressure.

Tubular permeability. The barrier to back-diffusion of urea has been treated as a thin membrane to which solutions on either side have free access. It is possible from the data to make an estimate of the order of magnitude of the permeability coefficient of this membrane, recognizing of course that the value is virtual rather than one to be identified with some particular structure. From the value it should be possible to decide whether or not the tubules present a unique barrier to back flow of urea.

In the derivation of the formula the product of permeability coefficient and total tubular surface area of the distal tubules was expressed as a single constant, k_2, for convenience in manipulation. Since trial of data from various sources (including unpublished experiments) has led to a value of about 0.3 cm.^{3}/min. for k_2, while the values of n and q_2 may be taken as approximately 2×10^6 and 10^{-2} cm.^{2}, respectively, we have

$$h_2 = \frac{k_2}{nq_2} \sim 10^{-5} \text{ cm./min.}$$

Jacobs (7) has given the permeability coefficient of ox erythrocytes for urea as 108×10^{-15} mols/μ^2/min./mol per liter. These dimensions, correctly expressed as mols/$\mu^2 \times$ min. $\times$ mol/liter, may be converted to the present dimensions of cm./min. if multiplied by the factor 10^{11}. This gives a value of about 10^{-2} cm./min. to be compared with the permeability in the order of 10^{-5}cm./min. estimated for the tubular wall. So far as comparison of such dissimilar systems is permissible, it may be inferred that the permeability of the tubular wall to urea is of a low order.

CONCLUSION

The behavior of urea clearance at varying volume flows of urine in human and dog kidneys is approximately described by assuming that urea diffuses from tubular urine to blood in two phases, related to the proximal and distal reabsorptions of water. Estimation of the permeability coefficient of the distal tubular wall for urea suggests that, as compared to ox erythrocytes, the wall has a low permeability.

The author wishes to express appreciation for the help given by Dr. D. D. Van Slyke in writing this paper and for the courtesy of Drs. H. Chasis and Homer Smith in making available original data from their paper (2).

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THE CHANGES OF IMPEDANCE OF THE TURTLE'S VENTRICULAR MUSCLE DURING CONTRACTION

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The electrical impedance of striated muscle increases during contraction (Bozler, 1935; Bozler and Cole, 1935; Dubuisson, 1937). In the turtle's ventricle, on the other hand, Rapport and Ray (1927) found a decrease of impedance during systole. The present study was undertaken to determine whether or not there is a fundamental difference between striated and cardiac muscle, in this respect.

METHOD. From pithed turtles the heart was excised. The several preparations of the ventricle used will be described with the corresponding results.

The measurements of the electrical impedance were made by means of an alternating current (a.c.) bridge. The muscle was in one arm of the bridge. It was balanced against a variable calibrated capacity (mica and paper condensers) and a variable non-inductive resistance, in parallel. The balance indicator was a cathode-ray oscillograph, after capacity-coupled amplification.

The balance was never perfect, thus showing that the electrical model used is only an approximation of the muscle. As shown by the records, however, the balance was sufficient to give a clear idea of the changes of impedance in the tissue.

A beat-frequency oscillator was used for generating the alternating currents delivered to the bridge. The frequency ranged from 30 to 10,000 cycles per sec. The results were qualitatively similar with all the frequencies tested. In the majority of the experiments 200 to 1,000 cycles were employed. The intensity (m.r.s.) of the current delivered to the bridge was from 0.04 to 0.06 milliampere (ma). The current flowing through the tissue was therefore about 0.025 ma. The electrodes were chlorided silver needles or else wicks wet with Ringer solution and connected to chlorided silver plates. The usual procedure was to balance the resting tissue. The changes of impedance during activity appeared then as an unbalance. These changes could be photographed from the oscillograph. The mechanical response was photographed simultaneously from an appropriate isometric optical myograph. The electrodes on the tissue were then connected with a direct-coupled amplifier activating the oscillograph and the ventricular electrogram was registered together with the mechanogram. Using the stimulus artifact, when the responses were stimulated, and the mechanogram, as common references in the two records, the electrogram could be compared with the changes of impedance.

A change of the balance of the bridge does not indicate the direction of the corresponding change in the tissue—i.e., whether the reactance or the resistance

[1] Fellow of the J. S. Guggenheim Memorial Foundation.

increased or decreased. Records were taken, therefore, in which the bridge was initially unbalanced in a known direction. A balancing or a further unbalancing change during activity gave then a precise indication of the change of impedance in the ventricle. Thus, if the resistance of the model was initially higher than necessary for balance, and if balance was restored during activity, an increase of resistance of the ventricle during contraction was indicated.

Results. It is important in the observations of the changes of impedance due to muscular contraction to prevent movement of the electrodes and shortening of the tissue. The following procedures were found satisfactory for those purposes and yielded consistent results.

Ventricular strips were cut in some instances. One end of the strips was stretched and fixed firmly on a board by means of 4 to 6 pins. The electrodes from the bridge were applied to this portion. The other end of the strips was attached to an isometric torsion-spring myograph.

In other cases the ventricle was cannulated through the atrium. The cannula was connected to a small membrane manometer. The system was filled with Ringer solution and was closed. The contractions were thus isometric. Needle or wick electrodes were applied to the external ventricular surface, or else one such electrode was used and another was in contact with the Ringer solution inside the closed system.

The most satisfactory method was to stretch the intact empty ventricle and fix it on a board by means of 6 to 10 pins, leaving a small basal part free. This free portion was attached to a torsion-spring myograph. The alternating current electrodes were applied near some of the fixing pins, to prevent movement, and near the mechanically recording part, to obtain a satisfactory temporal correlation between the contractions recorded and the changes of impedance. This method was used in the majority of the observations.

Although all these procedures tended to minimize movement, they did not prevent it entirely. Whenever movement did occur the results were inconsistent and complicated—an increase or a decrease of impedance could appear during contraction, or else an initial increase with a later decrease, or vice versa. The results, on the other hand, were consistent and relatively simple when no movement was seen at and between the leads. The description will deal only with these observations.

Typical changes of impedance associated with a response are illustrated in figure 1. In A the resting balance is disturbed reversibly. The beginning of the change coincides approximately with that of the mechanogram. The two curves are parallel at first but the impedance change outlasts the contraction. A comparison of A with C shows that there is no parallelism between the impedogram and the electrogram. At the end of the electrical response the impedance is still far from the resting value and only returns to that value later.

As explained under Method, the direction and characteristics of the changes of impedance were studied by recording with a known unbalance. If the resistance in the electrical model in the known arm of the bridge was too low, or if the capacity was set lower or higher than the proper value for balance at rest,

the unbalance was exaggerated during contraction. If the resting unbalance was obtained by increased resistance, however, then a relative balance appeared during the response (fig. 1B). It may be inferred that the impedance of the ventricle increases during its activity and that this increase is due largely to an increase of resistance, with the frequencies of alternating current used.

This inference was supported by measurements of the settings of the bridge which gave the best balance at the peaks of a series of contractions, and by com-

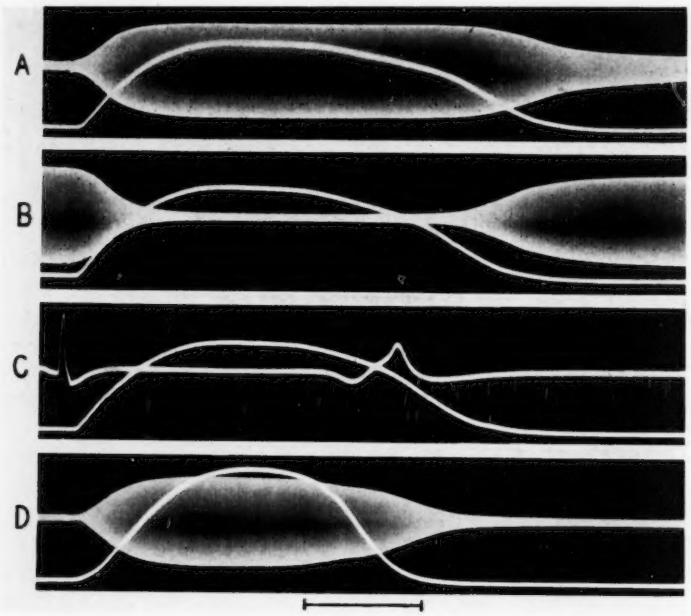

Fig. 1. Typical changes of impedance during contraction. Electrodes on two uninjured points in the ventricle. In this and other figures the lower tracing is the isometric mechanogram; the time calibration corresponds to 1 sec. The frequency of the alternating current was 500 cycles per sec.

A. Bridge balanced before stimulation. Settings of the known arm: resistance, 3130 ohms; capacitance, 0.0112μF. B. Bridge unbalanced by increasing the resistance to 3730 ohms. C. The upper tracing is the electrogram recorded from the same electrodes used for the records of impedance in A and B. D. As in A, but after warming the heart.

parison of these values with those corresponding to the resting balance. Thus, in a typical observation, with an a.c. of 0.04 m.a. and 500 cycles, the resting balance values were 3130 ohms and 0.011 μF. These values changed to 3480 ohms and 0.008 μF for balance at the peak of contraction. The impedance thus changed from 3120 to 3470 ohms.

As a rule the peak of the change of impedance coincided approximately with that of the contraction (fig. 1). Not infrequently, however, the maximum of the variation of impedance was later or, more rarely, earlier than that of the

mechanical response. It is difficult to rule out the possibility that an artifact due to movement may have determined the pattern of these atypical records. Impedograms with two peaks, early and late respectively, with reference to the maximum of contraction, were only seen when there was obvious movement of the tissue.

The duration of the impedogram varied with that of the mechanogram. Thus, when warming of the ventricle resulted in a brief contraction the variation of impedance was correspondingly brief (cf. fig. 1A and D).

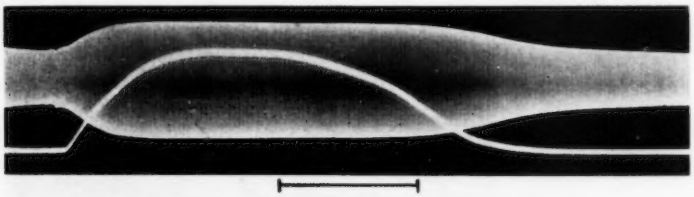

Fig. 2. As in figure 1A. Last response in a series of six, at the rate of 1 per 3.5 sec.

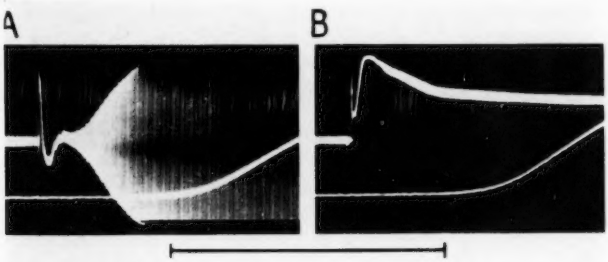

Fig. 3. Changes of impedance recorded from one electrode on intact ventricle, which was also the stimulating cathode, to another on a crushed region. Both these electrodes were at some distance from the portion of the ventricle which recorded mechanically, hence the long latency of the contraction. Only the beginning of the responses is reproduced.

A. Impedogram with about 4 times higher amplification than in figure 1. B. Electrogram recorded from the same electrodes.

Since the change of impedance outlasted the development of tension, it was possible to elicit a further response before an earlier one had fully subsided. A typical observation is illustrated in figure 2, which reproduces the 6th response in a series at the rate of 1 per 3.5 sec. The maximum variation of impedance was not greater than that corresponding to the 1st response of the series, but the subsidence of the change was slower than with single stimuli (cf. fig. 1A and fig. 2).

In order to determine whether there is any variation of impedance coincident with the beginning of the development of the electrical response, records were taken with high amplification and with one of the electrodes serving both as a stimulating cathode and as a connection to the bridge. As shown in figure 3 the electrical response began before the change of impedance.

DISCUSSION. As pointed out in the section on Method, the model of the tissue used—resistance and capacity in parallel—is quite imperfect. A perfect model would result in a perfect balance, which was not obtained here. Furthermore, with a perfect model, balance for a given condition of the tissue should not be modified by changes of the frequency and intensity of the alternating current. Such changes led invariably to unbalance in these observations.

The finding of an accurate model would involve more elaborate experiments than were carried on here (see Bishop, 1929; Cole, 1940). Notwithstanding the imperfections mentioned, the model was used because it is simple and because the measurements of the changes of impedance are accurate—i.e., if the impedance of the tissue is less than that of the model at a given time, more current will flow through the arm of the bridge containing the tissue than through the other, and vice versa.

We are unable to account for the discrepancy between our observations and those of Rapport and Ray (1927). The electrodes in their study were placed one in Ringer solution filling the manometer connected to the cannulated ventricle and the other in a solution bathing the external surface. We tried this method but discarded it because we could only record very minor changes of impedance. This difficulty was expected, for the arrangement is such that the ventricle is effectively in series with a relatively high resistance—that of the Ringer solution intervening between the tissue and the electrodes. We found further that twisting of the ventricle during contraction, or without a contraction, could result in marked changes of conductivity, due probably to changes in the degree of occlusion of the orifice of the cannula.

Since more than 100 observations in 20 hearts were consistent in showing an increase of impedance during activity, and since a decrease of impedance was only registered when movement was obvious at or between the electrodes, it is concluded that an increase is genuine and a decrease is an artifact.

Dubuisson (1937) has described two separate components in the impedance changes during contraction of striated muscle. These 2 components would result in 2 peaks in the impedogram, one during the phase of rising tension, the other during relaxation. Impedograms with 2 peaks were seen in these observations only when there was obvious movement, and hence an obvious source of artifact (p. 517). A complex impedogram might be expected because of the complexity of the ventricular response. Thus, it would seem likely that both the excitatory and conducting processes on the one hand, and the contractile mechanism on the other, would be associated with independent variations of the conductance of the tissue. The present data do not confirm this expectation.

In nerve, where there is no contractile mechanism but only excitation and conduction, Cole and Curtis (1939) have shown that there is a decrease of impedance associated with the passage of a nerve impulse. This decrease starts shortly after the beginning of the spike potential and reaches a maximum approximately at the same time as the spike. It was expected, therefore, that there would be a similar initial drop of impedance in the ventricle. The record in figure 3A does not confirm this expectation. If there is an initial drop it is very small, compared to the later rise of impedance.

The significance of the variations of impedance during activity can not be determined with the data available. Since these variations outlast the changes of tension, a recovery process is suggested (see Dubuisson, 1937). Whether this recovery is associated with conduction, or with contraction, or with both, remains to be elucidated. When the mechanical events are briefer the impedance changes are also briefer (fig. 1A and D), but, as is well known, the electrical responses are also shorter in these conditions.

SUMMARY

The changes of electrical impedance during the activity of the turtle's ventricle were measured by means of an alternating current bridge and recorded from a cathode-ray oscillograph.

The impedance to alternating currents of 30 to 10,000 cycles per sec. and of 0.04 to 0.06 ma. increases during activity. The increase begins approximately at the same time as contraction, but it outlasts the mechanical events (fig. 1A and B). There is no parallelism between the impedogram and the electrogram (fig. 1A and C). Brief electrical and mechanical responses involve a brief impedance variation (fig. 1D).

Upon repetitive stimulation a long enduring impedance change may ensue (fig. 2).

The early part of the ventricular electrogram is not attended by any striking change of impedance (fig. 3).

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A COMPARISON OF ATROPINE AND CURARE AS ANTAGONISTS OF ACETYLCHOLINE[1]

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The action of acetylcholine (Ach.) is usually considered to be nicotinic or muscarinic, depending on whether its effect is inhibited by atropine or curare, respectively.

Not all experimental data fit this formulation. Clark and Raventós (1937) observed *in vitro* that curarine and atropine blocked the effect of Ach. both for the auricle and the rectus abdominis of the frog. Marrazzi (1939) showed that in the rabbit the nicotinic effects (superior cervical ganglion) are antagonized by atropine. On the other hand, Luco and Meza (1941) established that curare blocked the connection between the parasympathetic postganglionic nerve and the smooth muscle.

Abdon (1940) stated that there was a constant ratio between the amount of Ach. necessary to produce a definite effect in the frog (heart inhibition, contraction of the rectus abdominis) and the amount of atropine capable of abolishing the effect. The object of the present work is to study in mammals the relationship between Ach. and curare. An attempt will also be made to interpret the differences found between the muscarinic and nicotinic effects of Ach. in the consideration of both groups of results.

METHODS. Cats were anesthetized with diallylbarbituric acid (Dial Ciba), 0.75 cc. per kgm. The response of the submaxillary gland was determined by the number of drops delivered from a glass cannula placed in Wharton's duct. The contraction of the superior rectus muscle of the eye was recorded on a kymograph with an amplification varying between 5 and 7, after removal of the eyeball. The muscle and the gland were decentralized by section of the common oculomotor nerve in its intracranial region and of the chorda tympani, and by extirpation of the ciliary and superior cervical ganglia. In some experiments the chorda tympani was stimulated with a Harvard inductorium. The injections were made into the common carotid artery. The drugs were from the following sources: acetylcholine hydrochloride (Hoffmann La Roche); atropine sulphate (Mallinckrodt); and curare (Merck), an empirical solution which was standardized biologically.

RESULTS. In all experiments the responses of muscle and gland were recorded simultaneously. The smallest amount of Ach. capable of inducing the minimum response of each effector was determined, using as end-points one or two drops from the gland and a definite movement of any part of the muscle. In each experiment the regularity of effect of the same stimulus was determined carefully, with injections of Ach. given at regular time intervals.

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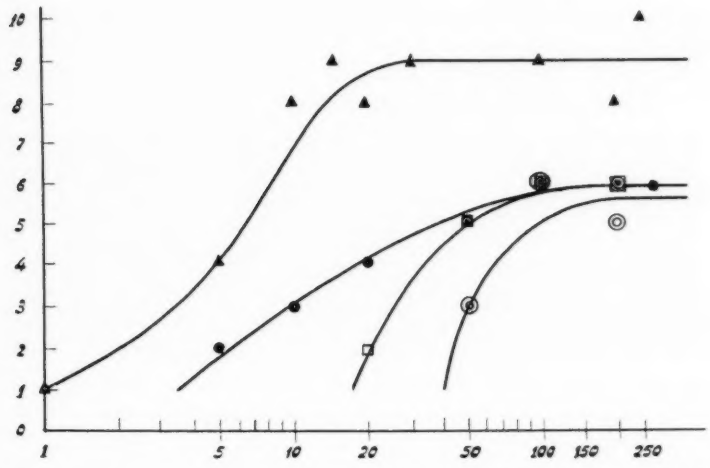

Fig. 1. Effect of curare on the response of the submaxillary gland to acetylcholine: Abscissae: dose of Ach. in micrograms (log. scale). Ordinates: intensity of response measured by the number of drops (arithmetic scale). Double circles, squares, filled-in circles: 1 minute, 17 minutes and 33 minutes, respectively, after injection of curare.

TABLE 1

The dosages of Ach. are the threshold values for each experiment; those of atropine and curare are the least amounts capable of blocking the responses to the respective threshold stimuli. Dosages are in micrograms. The letters M and G stand for muscle and gland. The designation — means that the corresponding dose was not determined. Dosage of curare is in cubic centimeters.

NO.	ACETYLCHOLINE		ATROPINE		CURARE	
	M	G	M	G	M	G
1	5	1	4,000	—		
2	10	1	11,000	—		
3	10	0.5	1,000	0.1		
4	10	1	6,000	1.1		
5	10	1	5,000	0.2		
6	5	1	4,000	1.0		
7	5	1	11,000	0.1		
8	5	1	6,000	1.0		
9	7.5	1			0.1	0.6
10	5	1			0.1	1.1
11	10	1			0.1	0.6
12	10	1			0.1	0.9
Average.....	7.708	0.958	6,000	0.583	0.1	0.80
Standard Deviation.....	±0.707	±0.041	±1,224	±0.170	±0.0	±0.122

1. *Atropine and skeletal muscle.* The effect of atropine on the response of the muscles of the eye to acetylcholine has been studied previously. Duke-Elder and Duke-Elder (1940) injected atropine intravenously (5,000 micrograms) and found that this drug did not affect the response in question.

Fig. 2. Antagonism between acetylcholine and atropine. Upper graph: muscle. Upper line: secretion of the gland in drops. Middle line: the numbers indicate the doses of Ach. (micrograms). *A* represents the injections of atropine: 0.1, 0.9, 1,000, 1,000 and 1,000 micrograms of atropine successively. Twelve minutes elapse between the two sections of the figure, during which 3,000 micrograms of atropine were injected. Time in minutes.

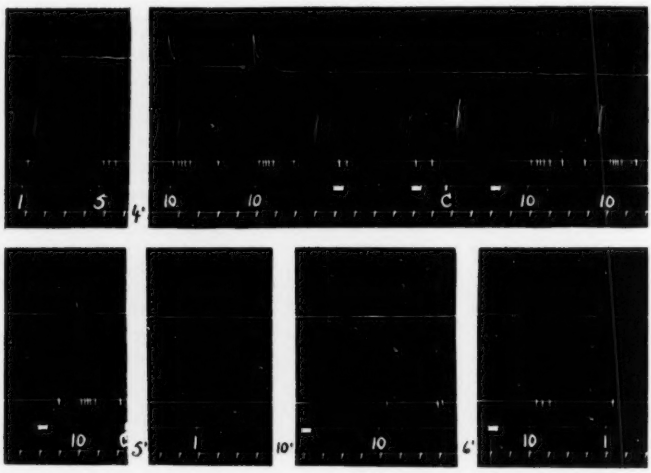

Fig. 3. Antagonism between acetylcholine and curare. Upper graph: muscle tracings. Upper line: glandular response. Intermediate line: the numbers stand for dosage of Ach. in micrograms; at the first *C*, 0.1 cc. of curare was injected; at the second *C*, 0.6 cc.; the other signal indicates the electrical stimulation of the chorda tympani. Lower line: time in minutes. The numbers between the lines indicate the elapsed time.

Our results indicate that it is inhibited by high dosages of atropine (fig. 2 and table 1).

2. *Curare and glandular responses.* Luco and Meza (1941) demonstrated that curare in large doses blocks transmission between the electrically-stimulated

parasympathetic postganglionic nerve and the corresponding effector; they suggested that curare inhibits all transmission of the cholinergic type, each system requring a different concentration. This hypothesis led to the supposition that the submaxillary gland, stimulated by Ach., could be curarized. Accordingly, the response of the gland as a function of the concentration of Ach. (intensity of stimulus) was studied in a group of animals, and also the modifications produced by curare. Figure 1 is a typical example of these experiments.

The normal curve of action-concentration is of the usual type. Curare raised the threshold to Ach. and limited the maximum response of the gland. Spontaneous decurarization is characterized by the enduring limitation of response, while the threshold returns to the normal value.

3. *Atropine and Ach.* During the period of stimulation with Ach., atropine was injected in varying dosage (between 0.1 and 11,000 micrograms, always in a volume of 0.2 cc.), noting the minimal paralyzing doses for the nicotinic and muscarinic responses, respectively. The results of each experiment are summarized in table 1.

If the amounts of atropine capable of inhibiting the responses of muscle and gland are compared, it will be seen that the doses are several thousand times greater in the first case than in the second (fig. 2).

4. *Curare and Ach.* In the same way as in the previous series, a comparative study was made of the minimum dose of curare necessary to block the effectors described above.

In this case, the results obtained were opposite to those of series 2, i.e., muscle (nicotinic effect) is inhibited by a dose of curare much smaller than that needed to inhibit the gland (muscarinic effect, fig. 3).

Discussion. A. *Technique.* The superior rectus muscle of the eye was used as an effector of the nicotinic type, and the submaxillary gland was selected as an effector of the muscarinic type, since they offer the following advantages over other effectors: 1. The extrinsic muscles of the eye are very sensitive to Ach. and thus the need for the injection of large doses is avoided, which may cause secondary effects. 2. The simultaneous stimulation of both effectors is very easy to carry out by means of intracarotid injections. 3. The short distance from the site of injection assures the constancy of the intensity of stimulus in both effectors.

Extirpation of the parasympathetic ganglionic system is very difficult, and therefore it was not done in any experiment. This renders difficult the interpretation of some results; Ach. not only stimulates the gland directly, but also in part through the ganglion. A similar argument applies to the inhibitions by both curare and atropine.

In order to evaluate the importance of the ganglion, a series of experiments were carried out in which the response of the gland was recorded following electrical stimulation of preganglionic parasympathetic fibers. Figure 3 shows a typical result in this series.

Stimulation of the chorda tympani induces a constant response of the gland. A small dose of curare blocks the response to an equal stimulus, without altering

the effects of a given dose of Ach. This demonstrates the slight importance of the ganglion in glandular stimulation by Ach.

B. *Results.* The observations cited in the introduction and the results of this work suggest the doubtful validity of the division of acetylcholine functions as muscarinic and nicotinic.

On inspection of the experiments with curare and atropine, it will be noted that the results are inverse, i.e., an effector with a high threshold for Ach. is blocked by large doses of atropine or small doses of curare; conversely, an effector with a low threshold is inhibited by high levels of curare or low doses of atropine.

If one accepts the general hypothesis that one or more processes occur between the stimulus and the response, these being the fixation of the drug and its selective action after fixation, as believed by Clark (1937), or that some other phenomenon may occur, the results described cannot be interpreted on a basis of there being only a quantitative difference between the muscarinic and the nicotinic effects. It is necessary to postulate at least the existence of two quantitatively different, but qualitatively equal, processes, and of necessity atropine acts on one and curare on the other.

The only other possible interpretation is that one or more processes are qualitatively different.

SUMMARY

The relationship between the acetylcholine-curare and acetylcholine-atropine antagonisms was studied in cats anesthetized with Dial as a function of the varying thresholds to acetylcholine exhibited by the superior rectus muscle of the eye and the submaxillary gland.

Large doses of atropine inhibit the muscular response to acetylcholine (fig. 2 and table 1).

Large doses of curare block the glandular response to acetylcholine (figs. 1 and 3, table 1).

The doses of atropine capable of inhibiting the responses to acetylcholine are directly proportional to the acetylcholine thresholds of the effectors used. With curare this relationship is reversed.

In the discussion, an explanation is offered for the differences between the nicotinic and muscarinic activities of acetylcholine.

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RESULTS OF PREFRONTAL LOBECTOMY ON ACQUIRED AND ON ACQUIRING CORRECT CONDITIONED DIFFERENTIAL RESPONSES WITH AUDITORY, GENERAL CUTANEOUS AND OPTIC STIMULI[1]

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This, the third study on the functions of the association cells of the cerebral cortex, is concerned with the effect of prefrontal lobectomy on acquired and acquiring correct conditioned differential responses of the foreleg from auditory, general cutaneous and optic stimuli. It will be seen that the results though negative in nature attain special interest when compared with earlier work on olfactory stimuli (1940) and with some preliminary unpublished work on these analysers after removal of large areas of the posterior cerebral association cells.

The term *correct conditioned differentiation* has been explicitely defined in earlier papers, in which the emphasis was placed on the correctness of the positive and negative conditioned responses rather than on fine discriminations. The same general procedures and controls were also used. The dogs represented the easily excited, easily inhibited and the evenly balanced type of cerebral cortex. Some of them 1, 3 and 4 were used in the previous report (1943), with which identical tests can be compared after prefrontal lobectomy. Ordinarily a two week recovery period preceded the first tests.

The results of these differential tests are summarized in tables and each table shows the records of two different conditioned differential tests for a common stimulant, one based on specific properties and the other on varied intervals of the same stimulant. In the first left column the dogs are listed by number, the first three being tested before and after prefrontal lobectomy and the others only after the extirpations. The second and third columns include the results of a single day's tests[2] after differentiation was standardized; the ratios of correct (C) to incorrect (I) for the positive conditioned tests appear in column 2, while the ratios of the incorrect (I) to correct (C) for the negative conditioned tests appear in column 3. Columns 4 and 5 are similar to 2 and 3 but include a record of all positive and negative conditioned reflex tests arranged for correctness and errors. A predominance of incorrect responses in these columns indicates difficulties encountered in acquiring correct differential responses. In these cases the negative tests ordinarily resulted in positive responses more often than the

[1] Aided by a grant from the John and Mary R. Markle Foundation.

[2] Usually 37 tests constituted a day's session for a dog. They consisted of 25 positive and 12 negative or the reverse and were given in varied ratios. A conditioned reflex was never reinforced except through punishment of an error. To be correct and avoid shock a positive reflex must occur within 7 seconds and to evade whipping or scolding a negative reflex must continue for 7 to 15 seconds, depending on the time of appearance of the positive reflex.

positive tests revealed an absence of response. The fifth or last column shows the grade of correct differential responses obtained.

Auditory stimulus. Three sets of differential tests were routinely used. In (1) a bell and a board tapped once per second served for the positive and negative stimuli. In (2) an iron cup was substituted for the board. In (3) the bell was tapped once per second for the positive reflex and three times per second for the negative reflex.

TABLE 1

Results of auditory stimuli after prefrontal lobectomy

DOG	BELL 1 PER SEC. POS. REF.		BOARD 1 PER SEC. NEG. REF.		DIFF.	BELL 1 PER SEC. POS. REF.		BELL 3 PER SEC. NEG. REF.		DIFF.
	A day's tests after diff. standardized		Total number of tests			A day's tests after diff. standardized		Total Number of tests		
	Pos. C to I	Neg. I to C	Pos. C to I	Neg. I to C		Pos. C to I	Neg. I to C	Pos. C to I	Neg. I to C	
Series 1. Tested before and after ablations										
1	24–1	0–12	65–1	7–81	good	12–0	3–22	60–1	9–41	good
3	32–0	1–9	176–12	46–60	good	24–1	2–11	100–15	19–23	good
4	12–0	0–25	60–2	2–46	good	13–0	1–24	140–5	30–81	good
						25–0	0–12			
Series 2. Tested after ablations										
2	24–1	2–8	148–4	43–24	fair	27–0	1–8	181–5	66–25	good
5	29–0	2–24	106–2	11–75	good	24–1	1–7	168–1	79–19	good
6	25–0	1–11	75–0	6–22	good	25–0	15–0	164–3	203–1	none
7	24–1	0–8	66–12	1–24	good	11–1	0–25	163–39	33–138	good
8	12–0	1–25	188–12	29–195	good	25–0	0–12	130–0	16–57	good
	25–0	0–12								

Abbreviations used in table

Diff. = Correct conditioned differential responses.
Pos. ref. = Positive conditioned reflex; Neg. ref. = Negative conditioned reflex.
Pos. = Positive conditioned tests; Neg. = Negative conditioned tests.
C to I = Ratio correct to incorrect; I to C = Ratio incorrect to correct.

It is clear from columns 2 and 3 of the left half of table 1 that dogs 1, 3 and 4, which were able to respond correctly with the conditioned differential tests with the bell and board before prefrontal lobectomy (table 1, previous paper), were still able to make a perfect or practically perfect score of a day's test after prefrontal lobectomy. The total number of tests (columns 4 and 5) also discloses a good percentage of correctness for dogs 1 and 4 after prefrontal lobectomy During the first session after operation dog 1 made 4 errors in 25 negative tests and none in 12 positive tests, while dog 4 made 2 errors in 25 positive tests and none in 12 negative tests. The first positive and negative tests were correct for both dogs. Dog 3 did very well for the second day's ordeal, then poorly for 2 sessions, to become practically perfect afterward.

The second group of dogs 2, 5, 6, 7 and 8 not subjected to any differential tests until after prefrontal lobectomy, reveals (columns 2 and 3 of the left half of table 1) smilar daily records for the bell and board to the group which was trained before the operation. The fact that the total number of tests (columns 4 and 5) discloses a general tendency toward correct responses for dogs 5, 6, 7 and 8, while dog 2 shows none, is to be attributed to a longer learning period for this dog. The number of daily sessions required of dogs 2, 5, 6, 7 and 8 before the first signs of differential responses appeared were 4, 2, 3, 1 and 1 days respectively. Since it required 2, 1 and 1 day's testing for the first group to

TABLE 2

Results of general cutaneous stimuli after prefrontal lobectomy

DOG	WITH HAIR GRAIN 1 PER SEC. POS. REF.		AGAINST HAIR GRAIN 1 PER SEC. NEG. REF.		DIFF.	WITH HAIR GRAIN 1 PER SEC. POS. REF.		WITH HAIR GRAIN 3 PER SEC. NEG. REF.		DIFF.
	A day's tests after diff. standardized		Total number of tests			A day's tests after diff. standardized		Total number of tests		
	Pos. C to I	Neg. I to C	Pos. C to I	Neg. I to C		Pos. C to I	Neg. I to C	Pos. C to I	Neg. I to C	
Series 1. Tested before and after ablations										
1	24–1	0–12	110–2	0–73	good	25–0	1–11	160–0	18–121	good
3	25–0	0–12	149–2	22–85	good	25–0	0–12	124–1	21–79	good
4	13–0	0–25	63–0	4–45	good	25–0	0–12	39–0	1–36	good
Series 2. Tested after ablations										
2	24–1	3–9	106–1	25–35	fair	22–3	0–12	46–4	1–16	good
5	1–11	25–0	34–57	219–29	inver.	25–0	0–9	46–4	1–14	good
7	24–1	1–10	176–22	28–108	good	11–1	0–25	139–9	21–102	good
8	25–0 12–0	0–12 0–25	108–4	5–68	good	25–0	0–12	37–0	0–37	good

Abbreviations used in table

Diff. = Correct conditioned differential responses.
Pos. ref. = Positive conditioned reflex; Neg. ref. = Negative conditioned reflex.
Pos. = Positive conditioned tests; Neg. = Negative conditioned tests.
C to I = Ratio correct to incorrect; I to C = Ratio incorrect to correct.

demonstrate the first signs of correct differential responses before prefrontal lobectomy, this group of dogs required but slightly more training than normal dogs.

After prefrontal lobectomy both groups of dogs made as high scores for a day's session with the bell and cup as they did with the bell and board and this was accomplished with the first or second day's tests. This excellent record with a similar but more difficult discrimination was obviously due to having followed the bell and board tests.

The results of both groups of dogs with the third set of sound analysers, namely, bell tapped once per second and three times per second are recorded

in the right half of table 1. Columns 2 and 3 show that with the exception of dog 6 both groups of dogs ultimately made as perfect daily records with this set of analysers as with the bell and board. After making due allowance for previous training, group 1 made a much better total record for correct responses than group 2 (columns 4 and 5). Dogs 1, 3 and 4 acquired the first signs of correct responses for the slow and fast bells in 2, 1 and 2 days of testing before prefrontal lobectomy and afterward in 2, 3 and 1 days respectively; while dogs 2, 5, 6, 7 and 8, not tested until after operation, required 6, 7, ?, 4 and 1 day of testing. Dog 8 while showing signs of correct differential responses during the first day's ordeal revealed no further signs of correct responses until the fourth day's tests. Dog 6 in not being able to respond correctly in 11 days of testing was unable to hold back his positive foreleg responses during 203 negative tests followed by punishment and intermixed with 167 positive tests, three of which were incorrect and punished.

Not only were all the prefrontal lobectomized dogs able to respond correctly to all three sound tests in varied ratios, but they were able to respond correctly to positive and negative conditioned tests alternated 4 to 6 times with intervals of 3 seconds between tests.

A more difficult sound conditioned differential test was attempted toward the end of the problem, unfortunately with but one dog and only after prefrontal lobectomy. The test was somewhat similar to one used successfully by Babkin with unoperated dogs. The positive stimulus consisted of tapping a bell once, then a second's pause, followed by tapping the bell 3 times per second and the negative stimulus consisted of tapping the bell 3 times per second, a second's pause, followed by 1 tap of the bell. The dog used was no. 8, the most likely of the prefrontal lobectomized dogs to acquire correct responses with this differential test. Out of 225 positive tests 215 were correct and of 298 negative tests 176 were positive and incorrect in spite of a whipping for each error. Respiratory tracings taken during negative tests that resulted in positive responses afforded other examples of 2 irradiated cortical waves, one excitable and incorrect and two, inhibitory and correct.

General cutaneous stimuli. Two sets of differential tests were used for this sense. In the first, the hair of the back was stroked with a hand brush once per second with the grain and once per second against the grain for the positive and negative conditioned reflexes. In the second set, the hair of the back was stroked with the grain once per second and three times per second for the positive and negative reflexes. The same groups of dogs were used in these tests as were used for sound. Group 1 (dogs 1, 3 and 4) was trained to make correct differential responses before prefrontal lobectomy; while group 2 (dogs 2, 5, 6, 7 and 8) was not tested until after the operation. Table 2 follows the same general plan for recording the results as was used for sound in table 1.

Columns 2 and 3 of both halves of table 2 show that the first group of dogs was able after prefrontal lobectomy to make perfect or practically perfect scores with both sets of general cutaneous stimuli for a day's test. Both before and after prefrontal lobectomy these dogs required only 1 or 2 days' testing to elicit

correct foreleg responses for either set of differential tests. After prefrontal lobectomy only dog 1 required a second session to acquire correct responses and it was for the tests based on rate of the stroke. This accounts for the good showing of the total tests in columns 4 and 5 of both sides of table 2.

Likewise columns 2 and 3 and 4 and 5 of both sides of table 2 show that the second group of dogs 2, 5, 7 and 8 (not trained before prefrontal lobectomy) have obtained perfect daily records for both general cutaneous tests after the operation and that a large number of the total tests were correct. The earliest signs of correct differential responses for the first set of general cutaneous tests (with and against the grain) appeared during the first day's tests with dogs 7 and 8, during the second with dog 2, but not until the fourth day for dog 5 and then the positive and negative reflexes were inverted.[3] The first signs of correct responses for the second set of differential tests appeared during the first day's testing with all dogs of the second group. It should be mentioned while dog 8 demonstrated signs of correct differential responses during the first session it was not until the fourth session that correct responses reappeared.

Both groups of dogs with the exception of dog 5 (not tried) were able to respond correctly to both sets of general cutaneous tests when the positive and negative conditioned tests were alternated 4 to 6 times with intervals of 3 seconds between tests.

A third set of general cutaneous tests was made on dogs 1, 3 and 4 in which inhalation of chloroform and acetic acid vapors served for the positive and negative conditioned reflexes. In these experiments the olfactory tracts were severed to eliminate olfactory stimulation. These dogs which were able to respond about 75 per cent correct before prefrontal lobectomy responded equally well afterward.

Optic stimuli. Dogs 1, 3 and 4, previously trained to average about 75 per cent correct differential responses before prefrontal lobectomy (1943, table 4) with two different sets of optic analysers, namely, light contrasted with black screens and constant light against a flicker (1 per second) were able to maintain about the same average of correctness after prefrontal lobectomy.

Lesion and autopsies. The lesion started directly in front of the precruciatus sulcus (supraorbital of Langley) and if the olfactory tract was to be severed extended to the base of the skull. It involved no difficulties, since the only precaution was to avoid slanting the spatula caudad to include the motor projection fibers.

Dogs 4 and 8 have not been autopsied for the reason that they are being used for another problem. The brains of the others have not only been carefully

[3] After prefrontal lobectomy dog 5 acquired the positive conditioned reflex of stroking the hair of the back with the grain during the first day's tests. Throughout the first three days' sessions for differential reflexes with and against the grain, all of the positive and negative tests resulted in positive responses. During the fourth session practically all the positive tests resulted in absence of response and nearly all the negative tests continued positive. These inverted reflexes continued throughout the remaining tests in spite of a total of 57 shocks and 219 whippings.

studied, but transections have been made through the medulla stained after the Marchi method to show pyramidal degeneration.

Complete ablation of the prefrontal areas was obtained in every instance with the exception of 5 to 7 mm. of the caudal end of the orbital lobe, namely, the region immediately behind the interorbital fissure (Langley's fig. 1, i.or.). There was slight injury to the median cephalic surface of the left sigmoid gyrus of dog 2, but Marchi sections through the medulla of this dog disclosed only a few more than the normal number of degenerated fibers in the corresponding pyramid. The brains of the other dogs showed no injuries to the motor cortex, no more than the normal amount of degeneration in the pyramids and none of the dogs exhibited motor symptoms.

Discussion. Since prefrontal lobectomy did not prevent the rapid appearance and perfect performance of certain conditioned differential responses from several different auditory and general cutaneous stimuli in dogs that were trained before and after the operation, its effects are obviously very different from the effect previously reported for olfactory stimuli. These results though negative in nature are of equal importance to the positive results of this ablation on correct differential olfactory conditioned responses for they suggest a lesser independence on the prefrontal or anterior association and correlation cells for correct analysis, synthesis, associated memory and the correct use of inhibition and excitation. It may be noted in this connection that a few preliminary experiments indicate a greater use of the posterior association cells surrounding the auditory, general cutaneous and optic projection centers (a large part of the sensory cortex of Dusser de Barenne, McCulloch, Garol et al.) for correct auditory and general cutaneous conditioned differential responses.

It might be supposed since the positive conditioned stimulus was the same for all of the auditory, and for all of the general cutaneous tests that the last set of differential analysers used would be those in which correct responses would be obtained with the least number of trials. This happened to be true for the general cutaneous analysers but not for the auditory. It is not strange that dog 3 of the first group and dogs 2, 5 and 7 of the second group had more difficulty with some differential tests than with others. Why all of the dogs of the first group were not able to make correct differential responses with the first tests is difficult to explain. It cannot be due to faulty circulation or edema of the posterior association and correlation cells or the posterior inhibitory areas because the positive conditioned reflexes appeared with the first trials and they are contingent on the functioning of the motor cortex which is situated directly behind the lesion. While the early errors are chiefly shown in the negative conditioned tests, cortical inhibition is not absent, for its presence is demonstrated in respiratory tracings taken at the time of incorrect positive tests and some correct negative tests. What is lacking is *correct* inhibition. The immediate delay of correct responses may be due to something akin to shock of the entire association cell system.

It is apparent that these results are at variance with Babkin's (quoted by Pavlov and by Eliasson) in which it was found that prefrontal lobectomy elim-

inated the positive conditioned cutaneous reflex from all parts of the body except the limbs.

It is singular in view of the results of others that Ten Cate was not only able to obtain conditioned sound reflexes but some differentiation between sounds after complete removal of the neopallium in the cat. It is however possible that the afferent and efferent connections between the cerebrum and the thalamus as indicated by Dusser de Barenne et al. and by Morison and Dempsey also contribute to the higher cortical activities.

SUMMARY

With one exception extirpation of both prefrontal areas produced no permanent effect on acquiring or on acquired correct conditioned differential responses elicited from somewhat similar tests with auditory, general cutaneous and optic stimuli.

Correct conditioned differential reflexes were also obtained from both groups of dogs after prefrontal lobectomy from sound and general cutaneous stimuli when the positive and negative tests were alternated 4 to 6 times with intervals of 3 seconds between tests.

After making due allowance for individual differences for certain tests and that the average dog had more difficulty in responding correctly to differential tests based on rate of a given analyser than on its various components, it is apparent that 2 of the 5 dogs, which were not tested until after prefrontal lobectomy, required more tests than normal dogs to respond correctly with the 1 and 3 second sound tests and one dog was unable to acquire the same in the time allotted for the tests.

The most promising of the second group of prefrontal lobectomized dogs did not acquire correct responses to a more complicated sound differential test.

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FACTORS INFLUENCING MICTURITION VOLUME IN THE RAT

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It is well known that after the fourth month of pregnancy, in human beings, the bladder increases in size and rises into the abdomen. During this period a condition of smooth muscle atonia develops which is made evident not only by relaxation of the bladder wall but also by relaxation of the ureters, uterus, large bowel and bile duct. Following parturition the bladder continues in a distended hypotonic state for a period of about six weeks, gradually returning to normal (1, 2, 3). Various theories have been advanced to explain these phenomena but little has been done to test the validity of the hypotheses.

Woolsey and Brooks (4) noted that marked increases in micturition volume occurred in cats post-partum. They also observed changes in micturition volume in cats during spontaneous estrus, during pregnancy and following injection of alpha-estradiol benzoate. Langworthy and Brock (5), using a different technique, found bladder hypotonicity to develop in the pregnant and post-partum rabbit and suggested that progestin might be responsible for the observed loss of bladder tone. We have attempted to obtain further evidence concerning the rôle played by hormonal factors in producing the changes in bladder responses known to occur during pregnancy and following parturition. Rats were used in this study because of their ability to rear litters at frequent intervals under ordinary laboratory conditions.

METHODS. Two techniques were employed in measuring bladder response and micturition volumes. In many instances both were used on the same animal to measure the changes in bladder reactions occurring during pregnancy and following parturition or hormone injections.

First, by means of kymographic tracings taken from the apparatus pictured in figure 1-A we were able to determine not only the total daily urine volumes but also the volume of urine eliminated during each micturition. The sensitivity of this device was such that 0.1 cc. of urine produced a 5 mm. excursion of the writing point. The daily water and food intake was measured; estrous cycles were followed and the animals were weighed once a week. Twenty-four females and four males were studied during many weeks and the effects of various hormones on bladder responses determined. Eighteen females were maintained in the apparatus throughout pregnancy and the subsequent period of lactation and nursing.

The second type of procedure permitted measurement of the bladder volume and intravesicular pressure at which micturition occurred in anesthetized ani-

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mals. We used an apparatus similar to that devised by Langworthy, Reeves and Tauber (6) and Schultz (7). After establishment of light ether anesthesia the rat's bladder was exposed and an 18 gauge hypodermic needle, connected by pressure tubing to the manometers and fluid reservoir, was pushed through the bladder wall and used to introduce warm saline into the viscus. Although the head of pressure was constant (90 cm. of water) the rate of bladder filling varied due to differences in bladder resistance to distention. Filling was continued until fluid escaped through the urethra. The volume and intravesicular pressure at which escape occurred were considered to be the characteristic micturition volume and pressure of that particular bladder. During each test period results

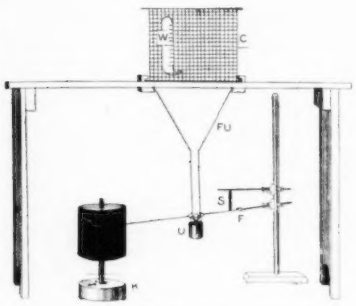

Fig. 1-A

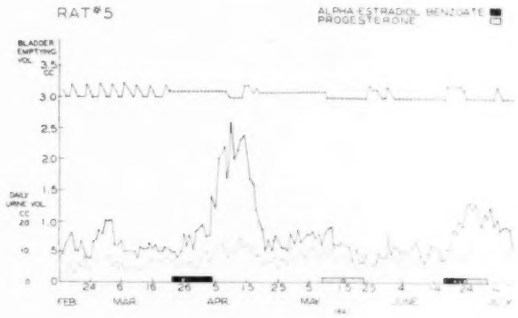

Fig. 1-B

Fig. 1-A. Apparatus for recording micturition volume of an unanesthetized rat. Cage, *e*, and water cup, *w*, placed on a stand above a funnel, *fu*, delivering urine to a cup, *u*, which is attached to a writing point recording on a slowly moving kymograph, *k*. The position of the fulcrum, *f*, and supporting spring, *s*, is such that very small increments in cup weight are recorded.

Fig. 1-B. Chart showing how average daily micturition volumes, average daily urine volume and estrous cycles of rat 5 were affected by injections of estrogenic material and progesterone. First injection—200 rat units daily for 12 days; 2nd injection—1 mgm. progesterone daily for 14 days; 3rd injection—200 rat units of estrogenic material for 7 days followed by 1 mgm. progesterone daily for 7 days.

obtained were checked by refilling the bladder to the micturition threshold four to six times. The hypodermic needle was then withdrawn and the incision closed. The same animal could thus be tested before pregnancy and before hormones were injected, during and after recovery from the effects of hormone injections and other procedures.

Results. 1. *Normal animals.* Results obtained on unanesthetized animals with the apparatus shown in figure 1-A revealed that, as in other species (2), each rat had a characteristic normal micturition volume. Female rats had, on the average, greater bladder volumes than did the males. Large rats tended to have larger bladder capacity than did smaller individuals but this was not invariably true. A comparison of the weight of the filled bladder to the body weight showed that the bladder comprised 0.15 per cent of the body weight in some individuals and 0.87 per cent in others. In the vast majority of cases the bladder normally

emptied when filled to such a volume that it constituted 0.25 to 0.30 per cent of the total body weight. In one group of six rats the individual body weights approximated 240 grams but their characteristic micturition volumes ranged from 0.42 cc. to 0.89 cc. The average micturition volumes of unanesthetized non-pregnant mature rats normally varied between 0.45 cc. and 0.65 cc. Some idea of the constancy maintained within the individual can be obtained from figure 2.

Anesthetics, or the procedures attendant upon the method of artificial filling, rendered the bladder much less responsive. When the same animals were used

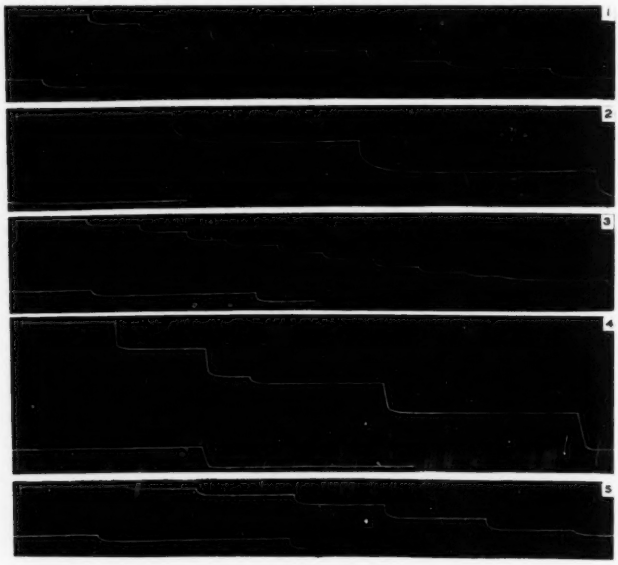

Fig. 2. Actual records taken with apparatus shown in figure 1-A. Records of rat 3 whose total history is shown in figure 3. (1) Normal—March 25. (2) Nursing—April 18. (3) Normal—May 16. (4) After estrogen injection—June 15. (5) Normal—July 20 (not shown in fig. 3).

which gave the above-stated averages in the unanesthetized state, micturition did not occur until a volume of approximately 0.9 cc. was reached. With this technique individual differences in the bladder tone of normal rats were revealed by the fact that despite a uniform head of perfusion pressure the rates of filling varied between 0.16 and 0.67 cc. per minute (average 0.43 cc.). The average intravesicular pressure at which micturition occurred was 30 cm. of water. Our results were similar to those obtained by Schultz (7).

2. *Pregnancy.* A study was made of bladder responses throughout sixteen pregnancies in a total of eleven rats. During pregnancy there was generally a gradual increase in micturition volume (fig. 3). The time of appearance of this rise varied enormously as did the magnitude of the changes. The rise began on

the second day in some individuals but in others it was definitely a terminal affair and was not detectable until the eighteenth or twentieth day following insemination. The maximal volume increment varied between 100 and 400 per cent of the average prepregnancy micturition volume.

These tremendous individual differences seemed to be related quite definitely to the outcome of the pregnancy. In the six animals which went through pregnancy and bore litters that lived and were nursed until the time of weaning, the changes in micturition volume during pregnancy were slight and occurred late. Changes in micturition volumes were detectable from the eleventh to the twentieth days and bladder size eventually doubled in some instances but this constituted the maximal change observed in this group. Micturition volume changes occurred earlier, between the fourth and eleventh day of pregnancy, in the five instances in which litters were not nursed and cared for effectively after birth

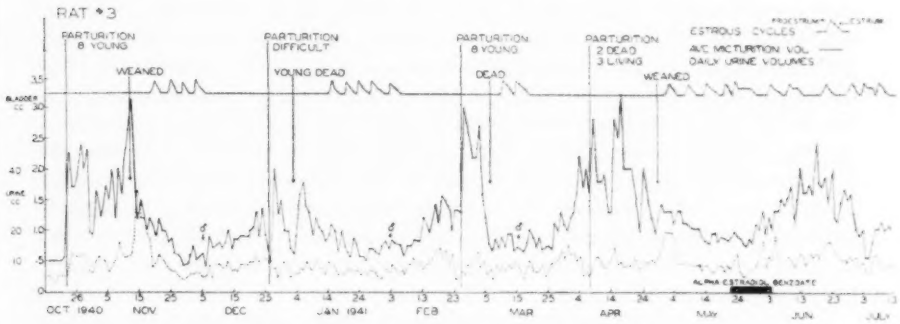

Fig. 3. Rat 3—chart showing effects of pregnancies which terminated normally and abnormally; effects of nursing and weaning, early death of young and injections of alpha-estradiol benzoate (200 rat units daily for 8 days).

(fig. 3). Five additional pregnancies were terminated by late (19 to 20 day) abortions or stillbirths and in these cases the rise in micturition volume had begun by the second or before the seventh day of pregnancy. The micturition volumes attained were three to four hundred per cent greater than the normal volumes for those individuals.

3. *Parturition and period of lactation.* Immediately following parturition there was an enormous increase in micturition volume which frequently attained a level of from two to nine times the normal. The average rise obtained in our series of animals was approximately five hundred per cent. In the group of rats whose litters survived until weaning the maximum micturition volume occurred from one to five days following parturition. These volumes then tended to decrease somewhat but still remained markedly elevated (about 3-fold the normal volume) as long as nursing continued. Within two to nine days following weaning the elevated micturition volume began to subside and reached a normal value in one to two weeks (fig. 3). Concomitant with the elevated micturition volumes there was occasionally a slight increase in the total daily urine output. In these

cases the daily urine output became elevated during the last third of pregnancy and remained elevated until the time of weaning. The total volume during this time was frequently three times the normal. During the first three days following weaning or preweaning-removal of the young there was a temporary rise of the total daily urine volume to a level which averaged five times the normal values (fig. 3). At this time the micturition volume was decreasing. Changes in bladder response consequently do not appear to be directly related to the volume of urine to be eliminated.

Five animals had litters which died within the first week following parturition. As was pointed out previously, a rise in micturition volume had begun rather early in pregnancy (4th to 11th day). In most instances the maximum micturition volume was reached shortly after parturition. The volumes remained elevated as long as the litters lived and usually remained high one or two days after death or removal of the young. The micturition volumes then fell precipitously and reached normal levels within two to ten days. In these animals the rise in total daily urine volumes was less marked than in the previous group and normality was attained immediately after loss of the litter. Comparison of the bladder changes observed to occur in these animals with those occurring when the litters were normally nursed, led us to conclude that lactation or the act of nursing prolonged the period of augmented micturition volume. To test this hypothesis a seven and a ten-day-old litter were removed from their normally lactating and nursing mothers. The two mothers were manifesting the typically increased micturition volumes. There was an immediate decrease in micturition volume and the bladder attained its normal prepregnancy level of response within a few days.

In five animals pregnancies resulted in abortion. The rise in micturition volume had begun very early in pregnancy and reached a peak at the time of abortion. The events, in all cases, were similar to those which occurred in rat 3. In this animal the initial micturition volume was 0.56 cc. Two days following mating the micturition volume began to rise, reaching 1.3 cc. on the 20th day. On the 22nd day of pregnancy the animal began to bleed. At this time the micturition volume rose to 2.2 cc., then dropped rapidly during the next seven days to 0.6 cc. On the seventh day following bleeding the animal aborted a single fetus, following which the volume again rose reaching the maximum value of 1.8 cc. before it began to fall again. This volume was reached on the tenth day. Thirteen days after the fetus was aborted the micturition volumes had returned to normal values. There was practically no rise in total urinary output at any time.

The results obtained from unanesthetized postpartum rats were confirmed by the technique of artificial bladder distention. For example, two nursing female rats were anesthetized and their bladders exposed and artificially filled with warm saline. In both instances repeated tests revealed that the bladder could be filled to a much greater volume than before pregnancy. In one case emptying volumes were 1.84 cc., 2.15 cc., 1.89 cc. as compared to 0.60 cc., 0.38 cc., 0.52 cc. observed before pregnancy and 1.01 cc., 0.79 cc., 0.70 cc. as obtained two weeks after weaning of the young. When exposed to a head of pressure of 90 cm. of

saline the bladder filled at a rate of 0.61 cc. per minute. This rate which was more rapid than normal indicated less tonic resistance to distention. The intravesicular pressure at which micturition occurred averaged 29.2 cm. of water, which is practically identical with the threshold pressure observed in normal rats. The trauma and irritation resulting from exposure and puncture of the viscus evidently caused hyperirritability of the bladder and a greater frequency of micturition. Micturition volumes decreased markedly after the test although they did not immediately attain the normal prepregnancy level.

4. *Injections of alpha-estradiol benzoate.* Twelve female and two male rats were given alpha-estradiol benzoate (Progynon-B, Schering) intramuscularly in amounts ranging from 0.0033 to 0.462 mgm. (20 to 2800 rat units) per rat, these amounts usually being given in divided doses over a period of 1 to 14 days. Total dosages amounted to from 0.1 to 13.9 rat units per gram of body weight. Two to six days following the beginning of such estrogen injections there was a rise in micturition volume similar to that seen in the pregnant animals (fig. 2). In four cases the rise did not begin until one week after termination of the injections. The maximum micturition volumes were attained in from 8 to 19 days after the initial rise became detectable and the peak volumes were from two to four times the normal values. After the maximum volume had been reached there was a progressive drop in the average daily micturition volumes. There was a great deal of variation but the normal characteristic micturition volume was again established within three to nineteen days (figs. 1-B, 3, 4). The two males injected with the estrogenic material showed similar changes though of lesser magnitude. In some instances the rise in micturition volume was accompanied by a two- to five-fold increase in the total daily urine output but there was no apparent relationship between magnitude of increase in fluid elimination and magnitude of bladder emptying volume. The rats varied greatly in their responsiveness to the estrogenic material. Some of the greatest changes in bladder tone resulted from the smaller injections (fig. 4). In two females there was no rise in micturition volume following injection of 0.066 to 0.28 mgm. (400 to 1700 rat units) alpha-estradiol benzoate per rat. It is possibly significant that these same animals likewise failed to show the usual picture of long continued estrus as determined by the vaginal smear method.

Acute experiments performed on six injected rats at the period of greatest micturition volume supported the previously described evidence that this estrogenic material modified bladder responses. The bladder did not contract and the sphincters did not permit escape of fluid until abnormally great volumes were attained. In one instance (fig. 4) the actual figures were 4.03 cc., 3.75 cc. and 3.8 cc. as compared to the animal's previously and subsequently determined normal threshold volumes of 0.83 cc., 0.75 cc. and 0.85 cc. The hypotonic condition permitted more rapid filling of the bladder than occurred before injection though the head of pressure was the same. The filling rate was 0.60 cc. to 1.2 cc. (average 0.88 cc.) per minute. The intravesicular pressure which resulted in opening of the sphincters averaged 27.3 cm. of water which was not greatly different from the average threshold micturition pressure of the normal rats.

5. *Alpha-estradiol benzoate followed by progesterone.* Three animals were given alpha-estradiol benzoate in doses ranging from 0.00079 to 0.0022 mgm. (4.8 to 13.9 R.U.) per gram of body weight, following which they were given progesterone (Proluton, Schering) intramuscularly in dosages ranging from 0.023 to 0.056 mgm. per gram of body weight. The estrogenic material was administered in daily amounts of 200 R.U. per rat for a period of four to fourteen days; the progesterone was given over a period of five to fourteen days, beginning five to twenty-one days after the estrogen injections had started. In one instance a single injection of 200 rat units of estrogenic material was bracketed by injections of progesterone (fig. 4). The observed rises in micturition volume resembled those resulting from the estrogenic material alone. The progesterone as administered did not seem to affect the micturition volume curve which follows injection of alpha-estradiol benzoate. The micturition volume began to rise

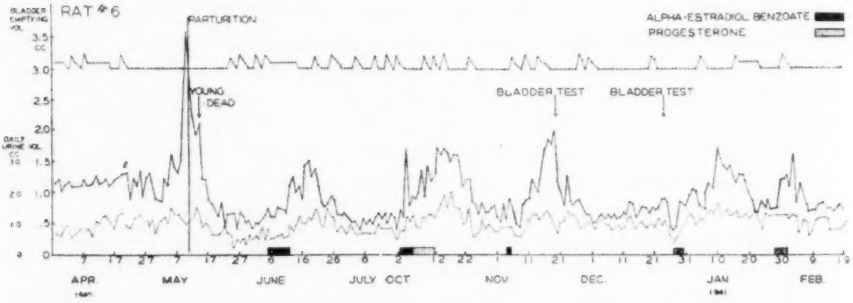

Fig. 4. History of rat 6. First injection—200 rat units daily for 8 days; 2nd injection—200 rat units daily for 4 days followed by daily injections of 1 mgm. of progesterone for 6 days; 3rd injection—20 rat units of estrogenic material for 2 days; 4th injection—1 mgm. progesterone for 5 days. On the third day 20 rat units of estrogenic material also given; 5th injection—2 mgm. of progesterone daily for 4 days.

two to eight days after the beginning of estrogen administration and the rise continued throughout the period of progesterone injection. Maximum micturition volumes of 1.3 cc. to 2.4 cc., representing a 2.2 to 2.8 fold rise in volume, resulted in 8 to 20 days following the onset of the estrogen injections. Recovery occurred and normal micturition volumes were reached between 25 and 33 days after the injections were first started. This showed no clear change from the picture obtained by giving estrogenic material alone (figs. 1-B, 3, 4).

6. *Progesterone.* Eight animals were given progesterone alone in amounts ranging from 4 to 28 mgm. per rat over a period of 4 to 14 days. Two of these rats were males. No definite change in micturition volume resulted. Such quantities of progesterone should have been adequate to affect the uterus and other tissues of the rat (8, 9). These injections did inhibit temporarily the estrous cycles of our animals but neither technique of estimating bladder response showed consistent variations from normal. We were unable to produce by means of this hormonal substance changes in bladder response similar to those observed

to occur in the pregnant and lactating rat. Figure 4 shows a case in which progesterone apparently caused a rise in micturition volume; figure 1-B shows a case in which the hormone seemingly produced a fall. In all other instances no changes were seen.

Discussion. Our measurements of bladder reactions in the rat consistently showed that during the last part of pregnancy and following parturition there is a tremendous rise in micturition volume. During this latter period a level of micturition volume is attained which is approximately five times greater than normal. This elevation is generally maintained following parturition until weaning has occurred. Early death of the young or their early removal also causes a precipitous decrease in micturition volume to the prepregnancy level. That these changes in the volume at which micturition characteristically occurs are due to changes in bladder muscle tone is indicated by several facts. Bladders of anesthetized postpartum rats can be filled artificially to an abnormally large volume before intravesicular pressure rises to a level which causes the sphincters to open. The intravesicular pressure which causes sphincter opening is practically the same in normal as in these atonic bladders. In seeking an explanation one of the most obvious assumptions to make is that the changes in bladder tone have a hormonal origin. Hormones which are known to affect the tone of certain smooth muscles are liberated in great quantity by the placenta during pregnancy and by the ovaries during pregnancy and lactation.

Langworthy, Kolb and Lewis (2) have suggested that progesterone might be the hormonal cause of the observed bladder hypotonia. Corpora lutea develop gradually during pregnancy as do the changes in micturition volume. Corpora lutea are also present during lactation and they degenerate at weaning. Corpora lutea are active during the periods of bladder hypotonia and they degenerate at a time when bladder recovery is occurring. There are, however, several phenomena which cannot be explained on the basis of progesterone production and disappearance. Our failure to reproduce the bladder changes by injections of progesterone blocks acceptance of the theory that this corpus luteum hormone is the sole cause of changes in bladder muscle tone. It might be, however, that some other corpus luteum hormone such as Relaxin is the agent modifying bladder response. We have no evidence either to establish or disprove this latter hypothesis other than the fact that bladder changes similar to those observed in the pregnant and nursing rat can be produced by injections of alpha-estradiol benzoate alone. It is known (9) that from 0.5 to 2.0 mgm. progesterone are required to produce detectable changes in a rat's tissues. The maximum doses which we used were only 14 to 46 times that amount. Estrogen injections, however, were from 20 to 2000 times the minimal effective dosage. It must be admitted that, on this basis of calculation, our progesterone dosages were much smaller in magnitude than were the estrogen injections. Even on this basis of comparison the smaller amounts of estrogenic material which caused bladder changes were less in magnitude than some of the progesterone injections which failed to produce an effect.

It is well known that progesterone alone may have little effect upon tissues but when preceded by estrogens it greatly modifies tissue responses. This applies

to uterine muscle (10). Uterine and ureteral muscle undergo similar changes during pregnancy (11). It consequently occurred to us that pretreatment of the rats with estrogenic material might reveal some effect of progesterone on the bladder. In the majority of experiments, however, progesterone, as given, seemed neither to add to nor detract from the characteristic estrogen effect.

During spontaneously occurring estrus in the cat a marked reduction in micturition volume occurs (4). Alpha-estradiol benzoate injections produce a similar drop in micturition volume. Following periods of spontaneous or artificially induced estrus, bladder volumes do tend to rise above normal but never as much as in the rat. Reactions of the two species during heat and during periods of estrogen injections are quite different. It may be that neural factors enter in to cause the species difference in bladder response. The postpartum bladder changes appear to be identical and since estrin seems to have opposite effects on the bladders of the two species this would favor the assumption that corpus luteum hormones rather than estrogens cause bladder hypotonia. This indirect evidence is not convincing in the light of more direct tests.

Lisco and Biskind (12) have claimed that high concentrations of estrone produce marked changes in the bladder wall and extreme dilatation frequently occurs. In our experiments on the rat injections of alpha-estradiol benzoate also caused a bladder hypotonus. We never observed a reduction in micturition volume in this species. Since injections of estrogenic materials produce changes in bladder response similar to those observed to occur following parturition one should consider the possibility that these postpartum changes are due to changes in estrogen production by body tissues. It is well known that the estrogen content of the urine rises during pregnancy then drops sharply after parturition (13, 14). One peculiarity observed in our experiments was that bladder changes frequently did not appear until after termination of estrogen injections. In a few rats the estrous cycle had been re-established before the rise in micturition volume had begun. This might be explained on the basis of a slow action of the hormone on the bladder. One could also hypothecate that hypotonia occurs most markedly in the bladder as recovery from estrogen modification is taking place. In the postpartum rat the greatest changes in the bladder occur as estrogen production is diminishing. The early large increments in micturition volume observed in pregnancies terminating in abortions and abnormal labor might be explained on the basis of abnormal placental function and early termination or diminution of estrogen production. The observations that nursing or lactation prolongs the period of bladder hypotonus and the sudden termination of suckling causes swift recovery of normal bladder tone are not easily explained on the basis of estrogen production or termination of such a production. It is possible that the ovaries in addition to producing more corpus luteum hormone during the lactation period are also forming estrogens in considerable quantity. The fact that estrogens tend to prevent lactation (14) argues against this assumption. We are not certain that variations in estrogen production are solely responsible for all observed changes in bladder tone.

It is frequently stated that abdominal operations tend to inhibit the bladder.

It has been suggested that the pain and trauma resulting from parturition might cause the postpartum retention. In the rat the changes are of such long duration that this explanation seems inadequate. Furthermore, exposure and puncture of the bladder during artificial perfusion experiments tended to reduce micturition volume (fig. 4). Pain and trauma may modify bladder reflexes but they are not the sole factors.

In some pregnancies bladder changes are rather slight and in some animals modifications of micturition volume do not follow administration of estrogenic material. Despite our lack of full understanding of the origin of changes observed to occur in the rat during pregnancy and following parturition and following hormone injections it seems reasonable to assume that hormones do modify bladder reactions. It is also clear that changes in micturition volume indicate changes in hormone production within the body.

These studies indicate that, at least in the rat, a slight and gradually developing decrease in frequency of micturition during pregnancy is normal. The average volume of individual micturitions may double. A great diminution in frequency and a great (400 to 500 per cent) increase in micturition volumes, however, indicate an abnormal termination of pregnancy.

SUMMARY

In rats there is normally a slight rise in micturition volume during pregnancy and a tremendous rise (averaging over 500 per cent) in the volume of individual micturitions following parturition.

There is a great variation in the rise in micturition volume in rats during pregnancy. This is correlated with the outcome of the pregnancy; i.e., in those animals whose pregnancies end in abortion or in early death of the litter, the rise begins quite early in pregnancy, whereas in those animals that give birth to living litters that survive until the time of weaning, the rise begins relatively late.

Following parturition the micturition volume remains high or is even elevated to a higher level. Micturition volume decreases somewhat during lactation but a normal frequency and volume of micturition is not restored until death of the young or until weaning.

Administration of an estrogenic substance (alpha-estradiol benzoate) produces an elevation of micturition volume which is similar to that seen during pregnancy and following parturition.

Progesterone, as administered in these experiments, does not produce any observable changes in bladder responses in the rat.

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EFFECTS OF EXTRACTS OF THE HYPOPHYSIS, THE THYROID AND THE ADRENAL CORTEX ON SOME RENAL FUNCTIONS

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We have reported (1) that diodrast[2] (D) and inulin (In) plasma clearances and maximum tubular output of diodrast (DT_m) are markedly decreased by loss of the anterior lobe of the hypophysis. The decreases in D and In plasma clearances were attributed to a decrease in renal blood flow and the decrease in DT_m not only to a decrease in renal blood flow but also to a depression of the functional capacity of the tubules to transport D at high plasma levels. These depressions of renal function were observed as early as 7 days after removal of the glandular hypophysis and they have persisted at the original level or decreased for more than two years. Because of the early onset of the change it was believed that the effect was probably direct rather than indirect through atrophic changes in other endocrine glands.

In this report evidence is presented to support this belief in that it is shown that an extract of the hypophysis of the sheep[2] markedly increases D and In plasma clearances and DT_m in normal, in simple hypophysectomy, in total hypophysectomy and in puncture dogs. Thyroid extract also increases these renal functions but to a lesser degree. Only in the puncture dogs does adrenal cortical extract[3] have any significant incretory effect.

PREPARATION OF ANIMALS. Trained adult female dogs were used as experimental animals. They were on a diet of one pound of raw horse meat daily with Purina dog chow ad lib. Operations on the hypothalamus or hypophysis were carried out through the oral approach. Simple hypophysectomy consisted in the removal of the glandular hypophysis and the posterior lobe. The median eminence was not removed. A dog so prepared does not exhibit diabetes insipidus and does not become markedly obese (0 to 20 per cent increase). Total or complete hypophysectomy consisted in removal of the entire glandular and neural hypophysis (median eminence included). Such a dog exhibits marked diabetes insipidus and becomes quite obese. "Puncture" consisted in the severance of the stalk and the removal with a special instrument of the median eminence and overlying tissues caudal to and above the median eminence. Such a dog exhibits marked diabetes insipidus and becomes markedly obese.

PROCEDURE. Previous to any hormone treatment, control experiments were

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[3] The adrenal cortical extract used in this research was supplied in part by the Upjohn Company.

performed on all animals to determine D and In plasma clearances and DT_{In}. After normal values had been obtained, hormone administration was begun and clearance experiments were run, usually on the seventh or eighth and on the fourteenth or fifteenth day after daily hormone administration. Adequate time was allowed for the animals to recover from any of the effects of the hormone therapy before another extract was given. Thyroid extract was given orally, 0.1 gram per kilo per day. In the case of the hypophysectomized animals, all of which showed more or less increase in weight, the dose was given on the basis of their preoperative weights. Such a dose will increase the basal metabolic rate 25 to 40 per cent in 10 to 14 days. Preloban Niphanoid[2] was given subcutaneously, each dog receiving 200 hypophysectomized rat units per day, with the exception of K17 which was given 100 units per day. Adrenal cortical extract[3] was given subcutaneously, some animals receiving 4 cc. per day and others 8 cc.

In the preparation of the animals for carrying out the clearances and the chemical methods for determining urine and plasma inulin and diodrast iodine, the procedures were identical with that described in a previous paper (White, Heinbecker and Rolf, loc. cit.). There were four normal, two simple hypophysectomy, two complete hypophysectomy and two puncture dogs in the series reported on. Of these one dog from each of the groups altered by an operation was autopsied to determine the state of the hypophysis or the hypothalamus. In every instance the examination of serial sections of the region involved indicated the correctness of the label attached to the designated group.

Results. *D plasma clearance.* The results are shown in table 1. Hypophysial extract produces a definite increase in clearance in all dogs tested. The average of three experiments on three normal dogs shows an increase from a base level of 317 cc./min./M^2 to 415 cc.; two experiments on 1 simple hypophysectomy dog, an increase from 124 cc./min./M^2 to 291 cc.; four experiments on two total hypophysectomy dogs, an increase from 179 cc./min./M^2 to 283 cc.; and two experiments on one puncture dog, an increase from 279 cc./min./M^2 to 361 cc. The effect is well established within seven days and the clearance remains elevated with continued administration of the extract up to 15 days. No attempt was made to extend the period of observation.

Thyroid extract produces an increase in clearance in all dogs tested but in general the effect is not so marked as that produced by hypophysial extract. As with hypophysial extract the greatest percentage increase occurred in the simple and total hypophysectomy dogs. In these, renal function was diminished previous to hormone therapy. The average of six experiments on three normal dogs shows an increase from a base level of 362 cc./min./M^2 to only 378 cc.; two experiments on one simple hypophysectomy dog show an increase from 124 cc./min./M^2 to 211 cc.; two experiments on one total hypophysectomy dog, an increase from 153 cc./min./M^2 to 235 cc. and two experiments on one puncture dog, an increase from 279 cc./min./M^2 to only 292 cc.

Adrenal cortical extract either left the clearance unchanged or showed a tendency to decrease it with the exception of the puncture dogs in which there was

an increase. The average of two experiments on two normal dogs showed a variation from a base level of 284 cc./min./M² to 281 cc.; two experiments on two simple hypophysectomy dogs a variation from 116 cc./min./M² to 105 cc.; four experiments on two total hypophysectomy dogs a variation from 179 cc./min./M² to 162 cc. and one experiment on one puncture dog, an increase from 279 cc./min./M² to 324 cc.

In plasma clearance. The results are shown in table 1. Hypophysial extract produces a rise in inulin clearance in all dogs but not to so marked an extent as was observed in the D plasma clearance. The average of three experiments

TABLE 1

	D CLEARANCE			In CLEARANCE			DT_m		
	cc./min./M²			*cc./min./M²*			*mg.I/min./M²*		
Normal:									
Before extract	317	362	284	95	110	83	21.2	20.8	15
After extract	P	T	A	P	T	A	P	T	A
	415	378	281	123	121	107	34.1	30.8	12.3
Simple hypophysectomy:									
Preoperative	279	279	273	83	83	79	14.8	14.8	15.2
Postoperative, before extract	124	124	116	50	50	50	2.4	2.4	2.4
After extract	P	T	A	P	T	A	P	T	A
	291	211	105	84	73	47	11.1	11.1	2.2
Total hypophysectomy:									
Preoperative	353	344	353	120	119	120	17.8	12.3	17.8
Postoperative, before extract	179	153	179	68	65	68	6.7	8.8	6.7
After extract	P	T	A	P	T	A	P	T	A
	283	235	162	83	71	47	14.4	12.6	5.6
Puncture:									
Preoperative	No observations								
Postoperative, before extract	279	279	279	91	91	91	28.2	23.4	28.2
After extract	P	T	A	P	T	A	P	T	A
	361	292	324	131	102	116	34.9	27.6	26.6

P—Anterior pituitary extract; T—Thyroid extract; A—Adrenal cortical extract.

on two normal dogs shows an increase in In plasma clearance from a base level of 95 cc./min./M² to 123 cc.; two experiments on one simple hypophysectomy show an increase from 50 cc./min./M² to 84 cc.; four experiments on two total hypophysectomy dogs show an increase from 68 cc./min./M² to 83 cc. and two experiments on one puncture show an increase from 91 cc./min./M² to 131 cc. Again the effects are quite marked at the end of seven days.

Thyroid extract also produces an increase in In clearance but at the dosages employed the increase is not quite so great as that produced with hypophysial extract. The average of six experiments on three normal dogs shows an increase in In clearance from a base level of 110 cc./min./M² to 121 cc.; two experiments

on one simple show an increase from 50 cc./min./M² to 73 cc.; two experiments on one total show an increase from 65 cc./min./M² to 71 cc. and two experiments on one puncture show an increase from 91 cc./min./M² to 102 cc.

The effects produced by adrenal cortical extract on the In clearance are variable. Both simple and total hypophysectomy dogs showed some tendency toward a decrease, the puncture dogs showed an increase corresponding to the increase in D plasma clearance, thus indicating an actual increase in blood flow. The normal dogs showed an increase in In clearance without a corresponding increase in D plasma clearance, a result probably due to constriction of the

TABLE 2

Puncture dog K 18

DATE	BODY LENGTH	WEIGHT	AVERAGE URINE OUTPUT PER DAY	HORMONE ADMINISTRATION	D CLEARANCE	I CLEARANCE	DT_m
	cm.	*kgm.*	*cc.*		*cc./min./M²*	*cc./min./M²*	*mgm.I/min./M²*
12/30/40 pre-op.	85.4	14		none	250	82	13.4
10/13/41 237 days post op.		25.7	5,500	none	251	85	13.3
3/ 6/41		31.5	6,000	9 days thyroid extract	388	127	19.95
3/10/42		31		15 days thyroid extract	379	115	34.4
5/14/42		34	6,500	8 days anterior lobe extract	458	128	27.22
5/21/42		34.5		15 days anterior lobe extract	332	116	26.5
7/27/42	91.0	35.5	5,800	8 days adrenal cortical extract, 4 cc./day	340	116	9.95
9/29/42		37	7,000	8 days adrenal cortical extract, 8 cc./day	460	140	21.3
1/ 8/43 686 days post op.		36.2	5,600	none	462	129	18.6
3/26/43	91.0	38	5,500	none	306	100	28.96

glomerular arterioles. The average of two experiments on two normal dogs shows an increase from a base level of 83 cc./min./M² to 107 cc.; two experiments on two simple hypophysectomy dogs show a slight decrease from 50 cc./min./M² to 47 cc.; four experiments on two total hypophysectomized dogs show a decrease from 68 cc./min./M² to 47 cc. and one experiment on one puncture shows an increase from 91 cc./min./M² to 116 cc.

DT_m. The results are shown in table 1. Hypophysial extract produces a very marked increase in DT_m in all animals, a rise greater than that observed in any of the other functions tested. The average of three experiments on three normal dogs showed an increase in DT_m from 21.16 mgm. iodine/min./M² to 34.13 mgm.; two experiments on one simple hypophysectomy show an increase from 2.37

mgm./min./M² to 11.05 mgm.; four experiments on two total hypophysectomy dogs show an increase from 6.71 mgm./min./M² to 14.36 mgm. and one experiment on one puncture showed an increase from 28.17 mgm./min./M² to 34.89 mgm. In all cases in which the DT_m was tested after one week and after two weeks of daily hormone administration, the value at the end of two weeks was higher.

Thyroid extract produced a marked increase in DT_m in the normal, simple hypophysectomy and total hypophysectomy dogs and a small increase in the puncture dogs. The simple hypophysectomy type was the only one showing an increase equal to that produced by a hypophysial extract. The average of six experiments on three normal dogs showed an increase in DT_m from a base level of 20.75 mgm. iodine/min./M² to 30.79 mgm.; two experiments on one simple hypophysectomy an increase from 2.37 mgm./min./M² to 11.10 mgm.; two experiments on one total hypophysectomy an increase from 8.8 mgm./min./M² to 12.57 mgm. and three experiments on two puncture dogs an increase from 23.38 mgm./min./M² to 27.58 mgm.

Adrenal cortical extract left the DT_m almost unchanged with a possible slight tendency toward a decrease. The average of two experiments on two normal dogs showed a change from a base level of 15.02 mgm./min./M² to 12.33 mgm.; one experiment on one simple hypophysectomy a change from 2.37 mgm./min./M² to 2.22 mgm.; four experiments on two total hypophysectomy dogs a change from 6.71 mgm./min./M² to 5.56 mgm. and one experiment on one puncture dog a change from 28.17 mgm./min./M² to 26.6 mgm.

The results on one puncture dog K-18 are presented separately in table 2. They are unique in that the base line for D and In clearances and DT_m increased markedly with time after operation. Interesting also is the observation that this dog even though considered full grown at the time of the operation increased considerably in length postoperatively. It seems possible that in this dog the eosinophil cells of the hypophysis have increased in number or effectiveness over the normal. The results show that hypophysial extract, thyroid extract and adrenal cortical hormone increased certain renal function and renal blood flow but not to the same degree as in the other puncture dog of this series in which the base line remained at its preoperative level.

Discussion. One of the primary objects of this investigation was to test further the validity of the conclusion previously drawn (White, Heinbecker and Rolf, loc. cit.) that the glandular hypophysis exercises a humoral influence on renal blood flow and diodrast transport at high plasma levels. The results herein reported support the thesis in that hypophysial extract has been shown capable of augmenting D and In plasma clearances and DT_m in normal, in simple hypophysectomy, in total hypophysectomy and puncture dogs. Such augmentation is marked where there has been a depression due to the removal of the glandular hypophysis.

Because of the rapidity with which the depression of renal function was initiated after hypophysectomy, it was believed probable that the action of the hypophysial hormone was in large measure a direct one. Among the possible

indirect pathways for such an influence, that of the thyroid and of the adrenal cortex now have been explored. The results indicate that the thyroid gland, particularly in states where thyroid activity is depressed, is capable of augmenting those renal functions which are augmented by hypophysial extract. Certain evidence would indicate that in the intact animal the degree of its influence on the kidney is probably less than that of the glandular hypophysis. Total thyroidectomy depresses the basal metabolic rate at the most 30 to 40 per cent in six to eight weeks. The depression in general blood flow presumably is not greater than this. The 50 per cent or greater decrease in renal blood flow after hypophysectomy comes on within four to six days. Hypophysectomy depresses the basal metabolic rate in dogs 15 to 20 per cent. Again without doubt the depression in general blood flow effected thereby is much below the depression in renal blood flow associated with the loss of the hypophysis.

Additional support for the belief that normally the thyroid gland exercises relatively little influence on the renal functions herein studied or on renal blood flow comes from the finding that in puncture dogs where there is no renal depression regressive changes occur in the thyroid gland similar to those which occur after hypophysectomy. In puncture dogs the changes in the thyroid are secondary to a loss of basophil cells in the anterior lobe of the hypophysis which follows the hypothalamic injury (unpublished data).

The observed effects of cortical extract on renal function in the dog indicate fairly conclusively that the trophic influence of the hypophysis is not exercised through stimulation of the adrenal cortex. Supporting evidence that the adrenal cortex does not influence renal function has been presented by Barnett, Perley and Heinbecker (2) in their studies of a case of Cushing's syndrome due to an adrenal cortical tumor. In this patient D and inulin clearances and DT_m were normal.

Other evidence that the hypophysis exercises a marked influence on the kidney is found in the demonstration by Winternitz et al. (3) and by White, Heinbecker and Rolf (4) that one remaining kidney does not hypertrophy in the hypophysectomized animal. Barnett, Perley and Heinbecker (loc. cit.) also have shown that in acromegaly the urea clearance may be markedly elevated (up to 230 per cent of normal). These investigators hold that under certain conditions renal function studies can serve to measure the functional activity of the eosinophil cells of the glandular hypophysis.

SUMMARY

Subcutaneously administered extracts of the glandular hypophysis increase the diodrast and inulin clearances and the maximum excretion of diodrast at high plasma levels in normal, in simple hypophysectomy, in total hypophysectomy and in "puncture" dogs, i.e., those with a hypothalamic lesion of a type causing diabetes insipidus and obesity.

Orally administered thyroid extract causes a similar though less marked increase in the above renal functions.

Subcutaneously administered adrenal cortical extract does not increase but

tends rather to decrease diodrast clearance in normal, in simple hypophysectomy and in totally hypophysectomized dogs. It increases diodrast clearance in the hypothalamic puncture dog. Inulin clearance in normal, in simple and in total hypophysectomy dogs usually is decreased by it but occasionally there is a moderate increase believed due to constriction of the glomerular arterioles. It increases diodrast and inulin clearances in the hypothalamic puncture dog. Diodrast T_m is decreased in the normal and in all the types of experimental dogs studied.

The results are interpreted as indicating that in normal, in simple and in total hypophysectomized dogs anterior lobe extract and thyroid extract increase renal blood flow and also increase the capacity of the renal tubules to transport diodrast at high plasma levels. Adrenal cortical extract does not increase, probably decreases renal blood flow slightly. It does not increase the capacity of the renal tubules to transport diodrast at high plasma levels.

In dogs of the "puncture" group anterior lobe extract, thyroid and adrenal cortical extract increase renal blood flow. Anterior lobe and thyroid extracts increase the capacity of the renal tubules to transport diodrast at high plasma levels but adrenal cortical extract does not.

The great effectiveness of anterior lobe extract in increasing renal blood flow and the capacity of renal tubules to transport diodrast is support for earlier evidence indicating that the anterior lobe of the hypophysis exerts a trophic influence on the kidney.

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SLOWING OF THE HEART AS A CONDITIONED REFLEX IN THE RABBIT

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Reflex slowing of the heart in the human subject is demonstrable in response to pressure on the eyeball—the oculocardiac reflex—and to inhalation of irritating vapors which stimulate either the endings of the nasal branches of the 5th nerve, or the sensory endings of the pulmonary vagus. The oculocardiac reflex has been made the subject of successful conditioning in man by eliciting this reflex at the same time as sounding a bell (1). Since the rabbit exhibits so marked a cardio-inhibitory response to inhalation of ammonia, it was thought that this animal might prove to be a good subject for determing whether slowing of the heart could be conditioned in a lower animal as well as in man.

A special canvas sling was prepared to support the rabbit with its legs hanging down through holes. Straps over its back held the animal in a comfortable position and prevented too violent struggling. Zinc electrodes on the shaven legs which had been moistened with saline, made connection with a string galvanometer. The animal was so placed that it could not see the experimenter as he manipulated the galvanometer, and the room was quiet and in darkness except for the light necessary to watch and to record movements of the shadow of the galvanometer string. A tube from a bottle containing ammonium hydroxide terminated just beneath the rabbit's nose. By means of a foot lever the operator could let loose a stream of ammonia vapor from this tube, the vapor being forced out by air which entered the bottle under pressure.

Each rabbit was brought into the experimental room at the same time each day and was handled very gently to avoid frightening. In accordance with Pavlov's suggestion an animal was allowed to become accustomed to the presence of one experimenter, and the same person handled it throughout the entire procedure.

Twenty minutes' rest was allowed after placing the animal in position, since it was found that this period was sufficient for the animal to become relaxed and the heart rate steady, an absolutely necessary condition for the experiment. This could readily be established by counting the swings of the shadow of the galvanometer string visible to the operator. This preliminary procedure was repeated on three successive days in order to accustom the animal to the experimental setup. Photographic records were then taken to show the normal heart rate, the effect of the bell alone, and of the ammonia alone. The heart rate was determined as beats per second using the crest of the QRS complex as guides and making measurements with as great an accuracy as possible by means of dividers. The film was run at a rate of 4 cm. per sec., with a time marker indicating each $\frac{1}{5}$ of a sec.

Conditioning was begun by sounding the bell and releasing the ammonia simultaneously. In a preliminary experiment in which a stethoscope was used to count the heart beats, the bell was rung for 20 sec. and at the same time an open ammonia bottle was thrust under the animal's nose. After 50 such pairings there appeared to be a decrease of about 50 beats per min. to the sound of the bell without application of ammonia. The difficulty in recording accurately a heart rate of over 200 beats per min. by this method led to the use of the electrocardiograph, but the time of ringing the bell and the amount of ammonia inhaled had to be reduced in order to avoid bodily movements which interfered with the electrical recording of the heart beat. A further experiment in which the bell was rung for 1 sec. only gave slight but definite indication of conditioning. In the final experiments the bell was rung for 5 sec. and the ammonia was allowed to flow only until the shadow of the galvanometer string showed that the animal's heart rate had become definitely slower, a matter of about 3 sec. Five such pairings were made daily with a rest of at least 5 min. between trials. To test whether conditioning had been established, the bell was rung without releasing ammonia at the end of each 10 pairings. Conditioning was evident after 20 pairings, but the response after 30 was much more striking.

Seven young but fully grown rabbits were put through this routine, three females and four males. Two of them, both males, proved unmanageable. Their movements so interfered with the recording that even after 20 trials they had to be discarded. The results on each of the five rabbits which we were able to condition were essentially the same, whether the animals were male or female.

The normal heart beat, once the animal had settled down in the cradle, showed a slight degree of arrythmia, but in the quiet, darkened room the greatest variation from the mean for minutes at a time would be 12 beats per min., usually less. Average rates were 228, 234, 222, 217, each characteristic of a given rabbit on a given day. The effect of the bell alone at the beginning of experimentation was an immediate increase in heart rate. This was evident from watching the shadow of the galvanometer string, but was impossible to count on the photographic records because of the irregularities due to general muscular movements in this "startle" response. The effect of inhalation of a single whiff of ammonia at the beginning of experimentation was an immediate and striking slowing of the heart rate from some 225 beats to about 55 per min. The slow rhythm persisted for about 7 sec. when the rate began to pick up. It had returned to normal within another 15–20 sec.

After conditioning was established the response to the bell alone was as follows: an immediate irregularity of the heart beat with indications of an increase of 12–18 beats per min.; in less than 2 sec. this was followed by an abrupt decrease of at least 30–36 beats per min. each with a plainly marked QRS complex. This rate was 18–24 beats below the normal resting rate and therefore beyond the range of the normal arrythmia. The effect is perhaps more strikingly shown by timing the intervals between individual beats as in the following record of rabbit G:

Before the bell was sounded the interval was 0.26 sec. for 4 consecutive beats. Immediately following the first stroke of the bell the interval shortened to 0.16 sec., and then

apparently to 0.10, the 2nd QRS complex being difficult to make out. The 3rd interval lengthened to 0.28, the 4th to 0.33, and throughout the rest of the 5 sec. period while the bell was ringing the interval remained in the neighborhood of 0.37. The interval returned to 0.26 exactly 3.6 sec. after the bell stopped ringing and remained at approximately this figure. On the basis of beats per min. this means that the rate before and after the bell was 230 per min. while during the ringing of the bell it was 162, a decrease of 68 beats per min.

It must be emphasized that intervals between beats when ammonia was presented might be as long as 1.3 sec., whereas in the conditioned response the interval was never more than 0.4 sec. Nevertheless the time by which the interval lengthened during ringing of the bell alone after 30 pairings with ammonia, viz., 0.11 sec. in the protocol quoted above, was more than 5 times the maximum variation observed while the animal was at rest, viz., 0.02 sec., the latter, of course, being either a lengthening or shortening of the interval. This, therefore, was considered sufficient evidence that conditioning had been established.

Further proof that this was indeed a case of conditioning was afforded by use of the method of extinction. Each rabbit was at the end of the experiment exposed to the sound of the bell alone without ammonia for 20 trials. Electrocardiographic records taken at the end of this period showed only the short preliminary increase in heart rate or no change at all. There was no decrease in rate during the 5 sec. of bell ringing beyond a return to the normal rhythm with its possible variation of 12 beats per min. The conditioned slowing of the heart had become extinct.

SUMMARY

It is possible so to condition a rabbit as to slow its heart rate by using inhalation of ammonia as the unconditioned stimulus and the ringing of a bell as the conditioned stimulus.

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DISTRIBUTION OF CORTICAL POTENTIALS RESULTING FROM INSUFFLATION OF VAPORS INTO THE NOSTRILS AND FROM STIMULATION OF THE OLFACTORY BULBS AND THE PYRIFORM LOBE[1]

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The purpose of this study was to determine by oscillographic procedure the distribution of the afferent association fibers from the pyriform lobe of the dog to other parts of the cerebral cortex, and to record briefly some confirmatory work on the afferent fibers from the olfactory bulb to the pyriform lobe.

Anatomical connections from the olfactory bulb to the pyriform lobe and amygdaloid nuclei have been established by many investigators. Hasama (1934) obtained action potential spikes from the pyriform lobe as a result of inhalations of quajacol, indol and chinolin. These were not obtained after cocainization of the nasal epithelium. Adrian (1942) obtained strong potential waves from inhalation and insufflations of air into the nostrils and these waves were altered when the air included clove and asafetida vapors. From this he concluded that the olfactory organ could be stimulated mechanically and chemically. Magoun *et al.* have reported by abstract that potentials were obtained from the pyriform lobe of the cat following stimulation of the olfactory bulb. The writer modified somewhat the technique of Hasama and Adrian. Xylol, a more powerful olfactory stimulant than cloves and asafetida (1929), was used in addition, and mechanical stimulations through the trigeminal and vagus nerves were eliminated by a previous procedure (1928–9). The approximate time of stimulation of the nasal epithelium by a vapor was recorded in the middle tracing of each record (fig. 2, A) by vapor from a branched tube contacting a microphone.

Oscillograph records from the pyriform lobe (fig. 1, points *2*, *3* and *4*) taken at the time of insufflation of xylol, cloves, asafetida or room air always demonstrated conspicuous spikes within approximately one-fifth of a second from the time the vapor reached the nasal epithelium (fig. 2 A, top record, middle for microphone and bottom for time). Likewise single shock stimulation of the olfactory bulbs was followed in approximately 10 milliseconds by pronounced spikes (fig. 2 B). Ordinarily the spikes following bulb stimulation were the largest and those from the air insufflations were the smallest.

Stimulation of the *pyriform lobe* at points *3* or *4* (fig. 1) with single shock resulted in conspicuous spikes in approximately 3 milliseconds from the ventrolateral portion of the prefrontal area (fig. 1, points *8*, *9*, *10* and possibly *20*), but not from other cortical areas (points *5* to *7* and *11* to *19*). Stimulation of the pyriform lobe was followed by antidromic potentials in the olfactory bulb, but

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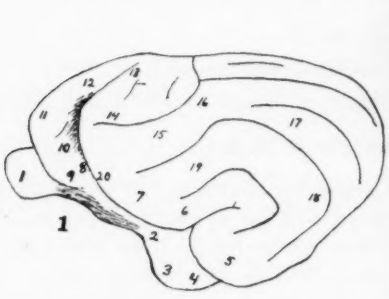
stimulation of the bulb did not alter the rhythm from points *8*, *9* and *10* from the prefrontal area (fig. 2 D). After under cutting the prefrontal area at points *8*, *9* and *10* from the rear in one dog, stimulation of the pyriform lobe no longer elicited spikes from the prefrontal area.



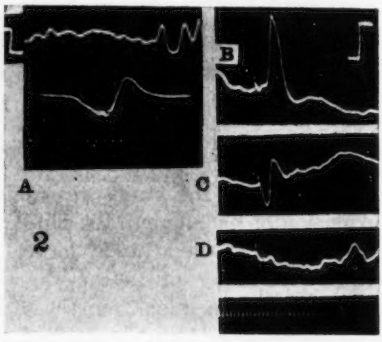


Fig. 1. Areas indicated by numerals represent points on a dog's brain which were stimulated or from which potentials were recorded.

Fig. 2. Oscillograph records in which an upward spike is negative. A. Top row, from pyriform lobe at time asafetida was insufflated into the nostril; middle row, simultaneous microphone record starting approximately at the time the vapor reached the olfactory epithelium; bottom row, 60 cps. 0.3 mv. B. From pyriform lobe during single shock stimulation of olfactory bulb. C. From prefrontal area (8) during stimulation of pyriform lobe. D. From prefrontal area (8) during stimulation of olfactory bulb. Records B to D, 200 cps. 0.3 mv.

Discussion. While Adrian's deduction may be correct that the olfactory organ is stimulated mechanically from air insufflations as well as from olfactory stimulating vapors, it is also possible that the keen olfactory sense of dogs may detect odors from inhalations or insufflations of air that cannot be detected by man. The spikes following our air insufflations were not elicited from trigeminal or vagal stimulation.

That potentials were recorded from the dog's prefrontal cortex (points *8*, *9* and *10*) following stimulation of the pyriform lobe is not surprising since it suggests the presence of association fibers corresponding to the uncinate fasciculus of higher vertebrates. The presence of association fibers from the pyriform lobe to the prefrontal area may explain the significance of these areas for obtaining correct conditioned differential foreleg responses with olfactory analysers (1940, 1941) and the relative unimportance of the prefrontal area for obtaining correct differential responses with auditory, general cutaneous and optic stimuli (1943).

SUMMARY

Action potentials from the pyriform lobe followed single shock stimulation of the olfactory bulbs or insufflations of xylol, cloves, asafetida or laboratory air into the nostrils of dogs in which trigeminal and vagal stimulation had been eliminated.

Single shock stimulation of the pyriform lobe evoked potentials from the ventro-lateral portion of the prefrontal area and antidromically from the olfactory bulbs, but not from other cortical areas. No spikes were recorded from the prefrontal area following stimulation of the olfactory bulbs or from stimulating the pyriform lobe of one dog after areas *8*, *9* and *10* had been under cut from the rear.

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THE INFLUENCE OF THE LIVER ON THE PROTEINS OF THE BLOOD PLASMA

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The association of disorders of the liver with the diminution of the total protein of the plasma was shown in clinical studies of the blood in hepatic disease by Grenet (1) and by Gilbert and Chiray (2). Numerous subsequent investigations have confirmed and extended these observations so that the liver appears to be a definite factor in the regulation of the protein content of the plasma. In the experimental animal Mann and Magath (3) found that after total removal of the liver there were a decrease of the concentration of albumin in the plasma and a tendency for the reversal of the albumin-globulin ratio. Similar observations have also been reported by Cantô (4) and by Franke, Toczyski and Lankosz (5).

Various other experimental conditions affecting the liver have been employed in studies of the plasma proteins. Kerr, Hurwitz and Whipple (6) found that the protein content of plasma of animals depleted by plasmapheresis was rapidly replaced by the formation of new plasma protein and that the globulin portion was replaced more rapidly than the albumin. Injury to the liver produced by the administration of chloroform or phosphorus resulted in marked delay of the regeneration of the plasma proteins lost by plasmapheresis. The extent of the delay of regeneration depended on the extent of the hepatic injury and in many experiments the time of the return of the plasma proteins to their normal level was coincident with the completion of repair of the injured hepatic tissue. The amount and character of the diet were also found to influence the curve of the regeneration of plasma protein. Jůrgens and Gebhardt (7) found an increase of the globulin of the plasma of Eck fistula dogs within two to three weeks after operation. Feeding of meat decreased the amount of albumin and increased the globulin present, and carbohydrate diets tended to maintain a more normal ratio. Sawada (8) found a decrease of the concentration of albumin and a slight increase of that of globulin of animals that had experienced hepatic damage following poisoning by phosphorus, chloroform or carbon tetrachloride as well as after inoculation with Schistosoma japonicum. In the hepatic damage following ligation of the common bile duct Bollman (9), Sawada (8) and Henriques and Klausen (10) found that the total protein of the plasma was decreased one to three months after operation. The albumin was markedly reduced and the globulin tended to be increased.

Similar reductions of the concentration of the serum protein have been observed in certain types of renal disease and in various types of malnutrition. That the fundamental cause of the alteration of the serum protein is not adequately explained either by the loss of protein from the body or by its inadequate intake has been discussed by Melnick and Cowgill (11). These authors indi-

cated that there is a failure of some mechanism of formation of plasma protein as well as the excessive loss or diminished intake of protein. By diet alone Holman, Mahoney and Whipple (12) caused alterations of the plasma proteins similar to those associated with diseases of the liver. Madden, Winslow, Howland and Whipple (13) found that formation of plasma protein may be greatly depressed in the presence of bacterial infection and inflammation. Under the influence of local inflammatory processes such as are produced by the subcutaneous injection of turpentine the regeneration of plasma proteins was only about a third as efficient as in the same animal with similar diets before and after production of the abscess. During this period the nitrogen balance was not altered to give indication of increased destruction of protein. Madden and Whipple (14) have published an excellent review of studies of the source, production and utilization of the plasma proteins.

In our studies we have determined the changes that occur in the protein content of the plasma after complete removal of the livers of dogs. The concentration of proteins of the plasma was reduced by plasmapheresis after hepatectomy and the influx of protein from the tissues other than the liver was determined by the subsequent alteration of the plasma proteins. Similar observations were made on normal dogs, and on dogs after procedures by which alterations of hepatic function were produced.

Methods. The animals used in these experiments were maintained on a standard diet consisting of 38 per cent ground lean horse meat, 35 per cent cracker meal, 15 per cent cooked tomatoes, 8 per cent lard and 4 per cent bone ash. This was fed once daily in amounts equivalent to twice the calculated basal caloric requirement of each animal. Variation of the protein content of the diet was accomplished by changing the meat content of the foregoing diet. Plasmapheresis was accomplished by withdrawal of blood equivalent to a fifth to a third of the estimated blood volume with subsequent reinjection of the erythrocytes. The blood was taken into a fifth volume of 2.5 per cent solution of sodium citrate, of which only traces remained after centrifugation and withdrawal of the plasma. The erythrocytes were washed once with saline solution and resuspended in saline solution prior to reinjection. When larger amounts of plasma were to be removed, additional bleeding and reinjections were repeated in the same manner. The total amount of serum protein removed was calculated from aliquot portions of each bleeding.

Removal of the liver was accomplished by means of the three-stage method. After operation the animals recovered from the ether anesthesia and were in good condition when plasmapheresis was begun one hour after completion of the operation. In some experiments blood was withdrawn and reinjections were made by direct puncture of an artery or a vein and in some experiments cannulation of the femoral artery was employed, procaine hydrochloride being used as a local anesthetic agent in the region involved. The concentration of sugar in the blood was maintained within normal limits by the continuous intravenous injection of 10 per cent solution of glucose.

The total protein, fibrinogen, euglobulin, pseudoglobulin and albumin of the

plasma were determined by Howe's modification of Kjeldahl's method, in which the nitrogen of the fractions is determined after precipitation within zones of increasing concentrations of sodium sulfate.

The determination of plasma volume, using the blue azo dye T 1824, has been made whenever possible, and the calculation has been made from this figure with the hematocrit value and the protein content of the plasma. Subsequent calculations assumed that the volume of circulating cells remained unchanged and the total blood volume was calculated on this basis. Other studies indicate that the volume of circulating cells as determined by the hematocrit varies with physiologic conditions. Because of increased cellular stasis after plasmapheresis, the calculated plasma volume is probably subject to considerable error and circulatory changes at different times may vary the extent of this error. Such errors, however, do not seem sufficient to alter the general trend of the results reported.

Results. Regeneration of plasma protein was studied after plasmapheresis of a third of the calculated blood volume under a number of different circumstances. The observed animals included normal animals, animals immediately after splenectomy, after removal of a third to a half of the liver, animals after alteration of the blood supply to the liver (Eck's fistula), and animals that had varying degrees of experimental hepatitis produced by repeated inhalation of carbon tetrachloride vapor. In most series, plasmapheresis was done after a preliminary feeding period of a week or more of three different diets. One diet consisted of lean meat, to another carbohydrate was added so the diet contained 12 per cent of protein and the third diet contained but 1 per cent of protein.

In all of the foregoing control experiments calculation of the data obtained after plasmapheresis indicated that some protein was added to the plasma during the first hour. Most of the addition was globulin in amounts up to approximately 1 gm. for each kilogram of body weight, and measurable amounts of albumin were also added. The greatest amounts of protein were added in the dogs which had received diets containing 12 per cent of protein, somewhat less was added in dogs receiving only meat and only 0.2 or 0.3 gm. of protein was added in animals that had received diets containing 1 per cent of protein. The Eck fistula animals and those that had extreme hepatic damage from carbon tetrachloride added only 0.1 to 0.2 gm. of protein for each kilogram of body weight and previous diet did not definitely influence this amount. Six to nine hours after plasmapheresis fibrinogen had returned to its original level or more in all animals except those that had severely damaged livers and the Eck fistula animals. Small amounts of globulin were added in all animals. Very small additions of albumin were detectable in dogs which had received diets containing 12 or 20 per cent protein except in the Eck fistula animals or those that had severe hepatic damage.

The data from which the foregoing conclusions were drawn were obtained immediately after ether anesthesia from animals which received dextrose intravenously during the period of experiment. The primary purpose of the experiments was to simulate the conditions which were employed in the animals after complete removal of the liver. The changes of the amount of circulating proteins

were calculated from the amount of plasma protein removed and from the total volume of plasma as determined at various intervals in the different experiments by the dye method. The main uncertainty of the calculations is the possibility that the sample of venous blood taken for analysis did not represent a true sample of entire blood of the animal. Stasis of blood in the capillaries probably occurs frequently in the experimental animals. While our results were consistent, it should be pointed out that the changes found were small. More ex-

TABLE 1

Plasma proteins after total removal of the liver

HOURS AFTER HEPATECTOMY	HEMATOCRIT	TOTAL PROTEIN	PER CENT OF TOTAL PROTEIN PRESENT AS			
			Albumin	Pseudoglobulin	Euglobulin	Fibrinogen
	per cent cells	*grams per 100 cc.*				
0	51.5	7.54	38.4	45.2	10.2	6.24
6	48.8	6.55	38.4	49.2	10.2	2.29
18	49.2	6.19	35.2	51.8	11.5	1.02
0	45.0	6.47	67.7	16.1	13.1	3.25
6	41.8	5.84	59.2	25.8	13.6	1.37
12	46.0	5.48	57.7	27.6	14.2	0.55
18	42.7	5.22	56.4	31.4	12.1	0.20
0	53.0	7.11	41.0	46.5	9.3	3.2
6	44.2	5.34	40.7	53.1	3.2	3.1
14	41.3	4.56	43.7	53.2	2.2	1.1
0	40.0	7.34	31.8	49.8	9.95	8.59
6	31.8	5.77	33.8	53.8	7.45	5.37
12	29.0	5.32	39.9	51.1	4.89	4.14
18	24.8	5.00	35.8	57.2	1.60	5.40
24	19.6	4.37	35.0	61.1	0	3.90
1	51.2	7.06	61.5	24.6	12.2	1.5
7	42.4	5.05	62.0	31.3	6.8	0
13	39.0	4.75	63.7	29.1	7.2	0
24	34.8	4.30	63.8	27.5	8.8	0
27	38.7	4.19	63.3	25.6	9.6	1.6
30	28.0	3.31	68.4	24.8	6.4	0.9

tensive plasmapheresis would be desirable, but we were unable to accomplish it in the dog after complete removal of the liver.

The effect of total removal of the liver. Because the greatest changes were found in the control animals receiving a diet containing 12 per cent of protein, all of the animals received this diet for at least seven days prior to total removal of the liver. The results of five typical experiments are given in table 1.

In all of these experiments some dilution of the proteins of the plasma occurred. In most cases the dilution of the proteins of the plasma was of the same order as

the dilution of the erythrocytes. This would suggest that the plasma protein was lost as whole blood, although the amount of blood in the peritoneal cavity at necropsy was not sufficient to account for this loss. Recalculation of the data so that the loss of plasma was proportionate to the loss of erythrocytes indicated that there was a loss (or dilution) of total plasma protein of 5 to 10 per cent. The loss of albumin was also 5 to 10 per cent while the loss of fibrinogen was 30 to 100 per cent. The loss of euglobulin was from 3 to 100 per cent in different experiments. The values for pseudoglobulin were usually increased, either a slight increase or as much as 66 per cent more than the original value. All of these changes occurred within the first few hours after operation. Subsequent analyses of specimens obtained up to thirty hours after removal of the liver showed only minor variations, but no major changes of protein distribution. Determinations of blood volume also indicated no great change of blood volume during this period.

The results of these experiments indicate that during the period of readjustment immediately following removal of the liver there may be an addition of pseudoglobulin to the blood. In no instance in these experiments did the addition amount to more than 0.5 gram for each kilogram of body weight of the animal. There is a somewhat irregular destruction or loss of fibrinogen and euglobulin but no appreciable change of the albumin of the plasma. In subsequent periods up to thirty hours there is no indication of loss or addition of proteins of the plasma.

Plasmapheresis after hepatectomy. In different experiments a third to a half of the total blood volume was withdrawn from the carotid artery of the dehepatized animal within a period of ten to twenty minutes. On completion of the bleeding an equivalent amount of washed erythrocytes from normal dogs was rapidly injected into the same artery. The erythrocytes were suspended in saline solution to the same volume as the blood withdrawn. Analyses were made on the first blood withdrawn. The animals were maintained by the continuous intravenous injection of 10 per cent solution of glucose in amounts sufficient to maintain normal values for blood sugar.

The results of two typical experiments of this series are given in table 2. In contrast to the hemodilution usually found in dogs maintained by continuous infusion of glucose after removal of the liver, hemoconcentration occurred following plasmapheresis. It is apparent that little protein is added to the blood of the dehepatized animal after plasmapheresis and that the colloidal osmotic pressure of the plasma is maintained by hemoconcentration. Calculation of the data on the assumption that concentration of the plasma has occurred equal to the concentration of erythrocytes indicates that some increase of the protein content of the plasma occurred during the procedure of plasmapheresis. In these experiments from 0.5 to 0.7 gram of globulin for each 100 cc. of plasma appeared to have been added during the fifteen or twenty minute procedure. The albumin content of the plasma was found to be very close to that expected from the withdrawal of the amount of blood and the dilution of the remaining blood with the saline suspension of erythrocytes. During subsequent periods, up to

nine hours after plasmapheresis, only minor changes occurred in the protein content of the plasma. There was usually a slight loss of protein from the plasma, which was probably due to a small loss of whole blood and subsequent dilution of the circulating plasma. In no case did there appear to be an appreciable destruction of albumin nor was there any replacement of this substance after total removal of the liver and plasmapheresis. In all experiments there was no loss of globulin and in a few there was a very slight increase of the globulin of the plasma several hours after plasmapheresis.

COMMENT. Some of the difficulties in the investigation of the effect of removal of the liver on formation of plasma protein have been mentioned. The necessary anesthesia, surgical procedures, loss of blood and administration of dextrose have some effect on the circulating blood. The samples of blood withdrawn from the larger vessels represent a true sample of the mixed circulating blood but give small indication of the state of the blood in the capillaries, which may be more

TABLE 2

Plasmapheresis after total removal of the liver

HOURS AFTER HEPATECTOMY	HEMATOCRIT	PLASMA PROTEIN	ALBUMIN	GLOBULIN	
	per cent	*grams per 100 cc.*	*grams per 100 cc.*	*grams per 100 cc.*	
1	31.3	7.80	5.40	2.40	Withdrew 40 cc. of blood for each kilogram of body weight and injected an equivalent amount of erythrocytes in saline solution
1¼	34.8	6.00	3.96	2.04	
10¼	44.7	7.00	4.50	2.50	
1	51.2	6.90	5.30	1.60	Withdrew 50 cc. of blood for each kilogram of body weight and injected an equivalent amount of erythrocytes in saline solution
1⅓	58.0	4.50	3.10	1.40	
7⅓	83.2	6.60	4.40	2.20	

concentrated or more dilute than the sample taken for analysis. This fact makes the interpretation of sudden changes hazardous, especially at the time of plasmapheresis. The calculation of the addition of protein to the plasma immediately associated with plasmapheresis is based on the increased proportion of globulin in the plasma beyond that which was withdrawn and at times an increase of globulin to more than the original level.

The fact that the changes due to plasmapheresis are present for several hours suggests that the observations are not due to a temporary change of concentration of the blood due to capillary stasis. The immediate influx of protein into the plasma during plasmapheresis may be due, however, at least in part, to the imposed alterations on the circulatory system as well as to the withdrawal of protein. Similar changes occur during the operation for complete removal of the liver. The only animals that did not add appreciable quantities of globulin to their plasma during plasmapheresis were Eck fistula animals, animals that had severely damaged livers and animals on diets deficient in protein. In all of these

the plasma albumin was definitely subnormal before plasmapheresis was done. Plasmapheresis after complete removal of the liver causes the addition of globulin to the plasma similar to that which occurs in normal animals.

Many of the questions concerning the rôle of the liver in the production of plasma proteins remain unanswerable. Many of the changes that occur in the plasma proteins coincident with removal of the liver are obviously due to causes other than the absence of the liver, since there is no consistent loss or gain with the progression of time following the operation. It would appear that in the absence of the liver the loss of protein from the plasma is too small to be measured by our methods within periods up to thirty hours after the removal of the liver. Plasmapheresis experiments after complete removal of the liver failed to indicate any regeneration of plasma protein within periods which were adequate for the demonstration of some regeneration of plasma protein in normal control animals.

SUMMARY

During the period of circulatory readjustment immediately following complete removal of the liver some globulin appears to be added to the plasma. Somewhat similar amounts of globulin are added to the plasma of well fed control dogs during circulatory readjustments associated with plasmapheresis. The addition of globulin is diminished or absent in animals that have Eck fistulas or severely damaged livers when the protein content of the plasma was previously abnormal. The immediate changes of concentration of the plasma proteins after complete removal of the liver are a small loss, or dilution, of total protein with a similar change of the albumin content, a marked loss of fibrinogen and euglobulin and an increase of the pseudoglobulin of the plasma. In subsequent periods up to thirty hours after removal of the liver there is little evidence of loss or addition of protein to the plasma. In periods up to nine hours after plasmapheresis in the liverless animal there is no evidence of regeneration of plasma proteins.

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THE EFFECT OF FASTING AND DEHYDRATION ON THE BLOOD SUGAR RESPONSE TO INJECTED INSULIN IN THE CHICK[1]

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It has been reported recently (1) that the chick offers possibilities as a test animal for the assay of insulin. Contrary to the general impression, the blood glucose of the chick is lowered in response to light doses of insulin and the duration of the hypoglycemia is shortened by increasing the dose. The percentage decrease of blood glucose 1½ hours after injection is found to be a straight line function of the logarithm of the dose up to about 1.5 units per kilogram. Although the individual variation in response is not excessive, attempts have been made to reduce this variation by prolonged fasting and dehydration. Some success has been achieved in this direction and, in addition, some facts concerning the effects of long fasting and dehydration on blood sugar and insulin sensitivity have been observed.

METHODS. Single comb White Leghorn cockerels were used throughout this investigation. They were received one day after hatching and placed in an electrically heated battery brooder and provided with Wayne Chick Starter mash and water at all times. The chicks were used between 30 and 40 days of age at which time the body weight varied between 200 and 400 grams. The average weight of each of the series was between 242 and 305 grams. Blood glucose was determined by the Hagedorn-Jensen method on 0.1 cc. of blood obtained from the wing vein (2). The dilution of the unmodified insulin[2] was made with acidified distilled water, pH 2.4, in such a way that the volume injected was always 0.5 cc.

The experimental animals are divided into two categories: group A consists of four series, each series being fasted 14, 24, 48 or 72 hours; group B contains three series, each series being deprived of both food and water for periods of 24, 48 or 72 hours.

Control blood sugars were taken in the afternoon following the fast, or fast plus dehydration, and the chicks immediately weighed and injected intramuscularly with 0.1875 unit of insulin. A second sample was drawn one and one-half hours after the injection. The same chick was never used more than once.

The nature of the results demand some measure of statistical analysis. Since the number of chicks varies from series to series, the significance of comparisons between series was tested by the expression: $M_1 - M_2/\sqrt{\sigma_{m_1}^2 + \sigma_{m_2}^2} = t$. The comparisons of variance were made by a modification of Fisher's Z test using the table of variance ratios (F) given by Snedecor (3). The values of F for the 5 per cent level of significance are given along with the data in table 2.

A total of 207 chicks was used in this study.

[1] Contribution no. 319 from the Department of Zoology.

[2] The insulin (Iletin) was supplied by Eli Lilly Co., Indianapolis, Indiana.

A. Control blood glucose and insulin sensitivity. *Results and discussion.* An inspection of table 1 shows that the blood sugars of both groups rose after prolonged fasting. In group A the control blood sugar increases significantly from 144 mgm. per cent after 14 hours' fast to 154 mgm. per cent after 24 hours of fast. Another significant rise occurs in this group between 48 and 72 hours of treatment. The 24 hour control value of 162 mgm. per cent found in group B, the fasted and dehydrated group, is significantly higher than the comparable control value of group A and the rise continues through the 48 and 72 hour series. The difference between the 24 and 48 hour series control blood sugars of group B is highly significant, but the difference between the 48 and 72 hour series falls short of significance. It is clear that the concentration of blood sugar increases both during fasting alone and with fasting plus dehydration. Dehydration would seem to have an augmentative effect and, at the

TABLE 1

The effects of fasting and dehydration on the blood glucose and insulin sensitivity of 30–40 day old chicks

TREATMENT	NO. OF CHICKS	CONTROL BLOOD SUGAR	1½ HOURS AFTER 0.1875 UNIT OF INSULIN	PER CENT CHANGE IN BLOOD SUGAR	AVERAGE WEIGHT
		Group A			
					grams
14 hour fast	30	144 ±2.1	118 ±2.4	−18.0 ±1.92	256
24 hour fast	31	154 ±2.0	128 ±3.9	−16.9 ±1.78	286
48 hour fast	30	159 ±2.4	125 ±3.0	−21.4 ±1.75	269
72 hour fast	19	175 ±3.3	140 ±3.4	−20.0 ±1.66	305
		Group B			
24 hour fast plus dehydration	38	162 ±1.8	136 ±2.8	−16.0 ±1.41	242
48 hour fast plus dehydration	29	179 ±2.0	139 ±2.1	−22.3 ±1.31	244
72 hour fast plus dehydration	30	184 ±1.9	154 ±2.8	−16.3 ±1.25	248

same time, cause a marked rise between 24 and 48 hours of treatment whereas, with fasting alone, this marked rise does not occur until after 48 hours of fasting.

The blood sugar response to injected insulin parallels the rise in control blood sugar very closely with one exception. The 48 hour series of group B had an initial blood sugar value of 179 mgm. per cent which fell to 139 mgm. per cent after insulin while the 72 hour series had only a slightly higher initial blood sugar but the drop in response to insulin was significantly less than that obtained in the 48 hour series. Upon comparing these two series it would appear that an insulin resistance had developed.

It is customary in mammalian work to express insulin sensitivity in terms of percentage change in blood sugar. The results of this investigation are also expressed in this fashion (see table 1), but since the duration of effect decreases with an increasing dose in the chick, no account can be taken of the time factor and the blood sugar level at 1½ hours after injection is used arbitrarily as the

measure of insulin sensitivity. These figures are very interesting. Apparent marked increases in sensitivity occur in the 48 hour series of each group and the 72 hour series of group A would appear to be more sensitive than the 72 hour series of group B. The greatest increase in response is in group B where the sensitivity changes from −16.0 per cent at 24 hours to −22.3 per cent at 48 hours. A similar rise is noted in the comparable series of group A. Both of these differences are significant. Again, comparing the 72 hour series of groups A and B, the 72 hour series of group A responds with a decrease of 20.0 per cent while the decrease of the 72 hour series of group B is only 16.3 per cent. This difference in sensitivity is probably significant, if account is taken of the considerable difference in body weights of the two groups (19 per cent). Unpublished data show that heavier chicks are slightly less sensitive to insulin than the lighter birds (4).

However, it cannot be stated with certainty that the fasted and dehydrated chicks are less sensitive after 72 hours of treatment than those which were only fasted. If the 14 hour series is taken as a reference point, then good proportionality exists between the control blood sugar level and the level after insulin injection of all series in both groups A and B. The greatest deviation from the expected value occurs in the 48 hour series of each group. In group A the expected value was 130 mgm. per cent, but a value of 125 mgm. per cent was observed; in group B 146 mgm. per cent was expected and 139 mgm. per cent found. However, these differences are slight and probably not significant, but it is interesting that they occur at a time when the control blood sugar is rising rapidly or on the verge of doing so. The blood sugar levels after insulin may be calculated in the same way for the 72 hour series of groups A and B. They are 143 and 150 mgm. per cent respectively. Using these figures in calculating the percentage change gives values of approximately −18 per cent for each series. Therefore, no significant difference would exist. The same is true in the other cases of apparent increased or decreased sensitivity.

An increase of blood glucose after 48 to 96 hours of fasting has been observed both in fowl and mammals (5, 6, 7). Selye reported (7) that rats are resistant to toxic doses of insulin after 48 hours' fast and also found a fasting hyperglycemia. These results are ascribed to an increased secretion by the adrenal cortex which would raise the blood sugar level and confer a non-specific resistance upon the animals, the "alarm reaction." Perhaps it would not be wise to press the comparison of chicks and rats too far, but some observations can be made. From the results presented here, it cannot be said that chicks exhibit an insulin resistance after fasting and in this respect they would seem to differ from the rat. The blood sugar of the fasting chick rises after 14 hours of fast and continues to do so for the duration of the experiment (72 hrs.). Again this is contrary to the finding on the rat under similar conditions where the blood sugar continues to decline until after 48 hours of fasting. If the prompt rise observed in the chick is to be explained entirely as a result of an "alarm reaction," then we must say that the development of this reaction occurs with considerable speed and that the extent of the reaction is increased considerably by dehydration.

An increase of blood sugar during fasting raises the question of the effect of fasting and/or dehydration on the utilization of carbohydrate. Evidence from mammalian sources (8) indicates that intact fasting animals do not readily utilize carbohydrate. There is also good evidence that the oxidation of carbohydrate is not impaired by fasting alone and the difficulty in disposing of administered glucose is due to the continued glucose formation from protein by the liver (9). Further, Golden and Long (10) have reported that the adrenal cortical extracts will raise the blood sugar and liver glycogen of the chicks as in the mammals. Considering these facts, a mechanism can be visualized whereby the chick can resist the hypoglycemic effects of prolonged fasting and insulin and may succeed in overcoming them to some extent. One might deduce that either the production rate of carbohydrate increases or the utilization rate decreases progressively after about the 14th hour of fasting. In either case, a rise in blood sugar occurs. The effect of this mechanism is such that sufficient carbohydrate is available to replace that which disappears after insulin injection and to prevent an increased sensitivity after prolonged fasting.

B. Comparison of variability of response. *Results and discussion.* The comparison of variance between experimental groups has two purposes: first, for the purpose of biological assay, it is desirable to use the least variable material providing that it is sufficiently sensitive, and second, the changes in variance under different treatment undoubtedly have a physiological significance and a knowledge of such changes may give a clue to the mechanism involved. Table 2 shows the comparisons made between the mean square deviations of groups A and B and between series within the same group. The variance in control blood sugar levels and levels after insulin injection must be considered, because the variance of the calculated percentage change in blood sugar presumably is a combination of these variances.

No significant variation is observed in the control blood sugar values of the three series of the fasted group, but there is a tendency toward greater variability with increasing length of fast. The chicks of group B, fasted and dehydrated, have perhaps a little less variation initially than those of group A and the variation tends to decrease with length of treatment. The divergence of the two groups is such that the difference in variance after 72 hours of treatment is of doubtful significance.

The behavior of the variance of the blood sugar values after insulin injection is rather peculiar. In Group A the variation within a series is least after 14 hours of fast, $s^2 = 171$, and the greatest after 24 hours when $s^2 = 470$. Thereafter the variance becomes progressively less. The ratio between the 14 and 24 hour series is highly significant. This is not true of group B where the *least* variance occurs after 48 hours without food or water. The variance of the 48 hour series as compared to the 24 hour series is significantly less (2.20) and that of the 72 hour series falls just short of being significantly greater than that of the 48 hour series. Upon comparing the two groups, A and B, it is apparent that only the 48 hour series are divergent enough to give a significant result.

The only point at which the variance of the calculated percentage change in

blood sugar is significant is at 48 hours. It is interesting to note that, in these data, the figures on the significance of the variance follows the value of the F ratio for the blood sugar values after insulin and not the F ratio of the control blood sugar comparisons.

TABLE 2

Comparisons of variability in blood glucose of fasted and fasted and dehydrated chicks before and after insulin

COMPARISONS BETWEEN	NO. OF CHICKS	CONTROL BLOOD GLUCOSE		BLOOD GLUCOSE AFTER INSULIN		PERCENTAGE CHANGE		VALUE OF F FOR 5% SIGNIFICANCE
		Variance (s^2)	F ratio	Variance (s^2)	F ratio	Variance (s^2)	F ratio	
24 hr. series								
Group A	31	144	1.12	470	1.59	100	1.28	1.76
Group B	38	235		295		78		
48 hr. series								
Group A	30	169	1.35	265	1.98	91	1.87	1.85
Group B	29	125		134		49		
72 hr. series								
Group A	19	214	1.93	223	1.06	52	1.16	2.07
Group B	30	111		237		60		
Group A								
14 hr. series	30	130	1.11	171	2.74	116	1.16	1.84
24 hr. series	31	144		470		100		
Group A								
24 hr. series	31	144	1.48	470	2.11	100	1.93	2.07
72 hr. series	19	214		223		52		
Group B								
24 hr. series	38			295	2.20	78	1.59	1.80
48 hr. series	29			134		49		
Group B								
48 hr. series	29			134	1.77			1.85
72 hr. series	30			237				

$$\text{Variance } (s^2) = \frac{\Sigma d^2}{n-1}.$$

The reason for the fluctuations of the variance under different treatment is not clear, but the trends indicate that the homeostatic mechanism is certainly disrupted in some cases and aided in others. There is reason to believe (1) that the stored carbohydrate in the liver is somewhat higher after 72 hours of fast than after 14 or 24 hours of such treatment. This may account for the increasing variability of the fasted group. Such an explanation, of course, must presume that carbohydrate is being manufactured from some endogenous source.

However, the blood sugar of the fasted and dehydrated group is higher than that of the fasted only group, yet the variability is markedly less. Unfortunately, no figures on stored carbohydrates for this group are available.

While no direct evidence can be offered at this time, it is suggested that these changes in variance represent changes in the fuel and operating economy of the chick. Once the stored carbohydrate levels have reached a minimum, protein, or fat, or both must be converted to carbohydrate in order to maintain the essential reactions involving this substance. Undoubtedly the differences in variance between the fasted and the fasted and dehydrated chicks have a physiological significance which remains for further elucidation.

SUMMARY AND CONCLUSIONS

1. Thirty to 40-day old chicks were subjected to fasting, or fasting plus dehydration, for periods of 14 to 72 hours and their sensitivity to 0.1875 unit of insulin was tested.

2. The control blood sugar rose after the 14th hour and continued to rise through 72 hours of fasting. Dehydration augments this rise. The blood sugar values of the fasted and dehydrated chicks rose more promptly and to higher levels than those of chicks which were only fasted.

3. No significant increase or decrease in sensitivity to insulin was observed in either group, although the 72 hour fasted and dehydrated group tended to be less sensitive than the 72 hour fasted group. It is concluded that neither fasting alone nor fasting plus dehydration affects the insulin sensitivity of the chick.

4. Fasting plus dehydration results in a marked decrease in the variability of blood sugar values. Fasting alone tends to increase the variability of control figures. The variance in blood sugar values 1½ hours after insulin injection is least in the chicks fasted and dehydrated for 48 hours.

5. The precision of the results after 14 hours of fast seems sufficient for routine insulin assay by the chick method.

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THE RELATION OF PHYSICAL EXERTION TO THE RESISTANCE OF RED BLOOD CELLS TO LAKING

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For a number of years it has been observed in this laboratory (1) that when a small amount of blood was introduced into distilled water, as in the first step of the volumetric method for the determination of lactic acid employed here (2), the resulting mixture showed various degrees of opacity. The opacity was greater when blood was drawn from subjects who had been running on a treadmill, and there seemed to be a rough relation between the degree of opacity and the severity of the exercise and the amount of lactic acid in the blood. Microscopic examination of the sediment after centrifugation revealed numerous red blood cells and shells of partially laked red blood cells. The unhemolyzed red blood cells which could be counted numbered between 500 and 1,000 per cu. mm. of the original blood. The introduction of plasma alone into distilled water did not produce opacity to a significant degree.

An empirical method of estimating the degrees of opacity was developed in order to study the phenomenon further and to compare the findings with the amount of muscular exercise and with lactic acid determination of the blood.

METHOD. Distilled water is brought to pH 6.8 by the addition of M/15 Na_2HOP_4. (In this laboratory, the distilled water requires about 1 to 1.5 cc. of M/15 Na_2HPO_4 per liter.) Brom-thymol-blue is used as an indicator (light blue color) employing Sorenson's Na_2HPO_4 and KH_2PO_4 buffers for control. The adjustment of pH is facilitated by employing the photo-electric colorimeter, filter no. 540.)

It is convenient to use capillary blood (collected by finger puncture) although venous and arterial blood give similar results; 0.2 cc. of blood is added to 10 cc. of the buffered distilled water in a colorimeter tube. Care should be taken not to bubble air into the solution while making the transfer. The contents are mixed gently. A drop of caprylic alcohol may be added if necessary. The mixture is allowed to stand 10 minutes before reading. It is probably inadvisable to allow the mixture to stand more than one hour.

The Evelyn photoelectric colorimeter with filter no. 660 has been used. With a tube containing 10 cc. of water the galvanometer thread is set at 100. The reading without the tube of water in place is noted. The blood mixture tube is inserted and a reading is made (G_1). One drop of NH_4OH is added, which clears the mixture. The tube is agitated gently, and a second reading is made immediately (G_2). The density values ($L = 2 - \log G$) for the corresponding galvanometer readings are obtained. The difference between L_1 and L_2 represents the "opacity" of the blood mixture. For example:

	G_1	L_1	G_2	L_2	$L_1 - L_2$ ("OPACITY")
1. Subject at rest	84	0.0757	90_1	0.0446	0.031
2. Same subject after run (2 minutes, 7 m. per hr., grade 8.6 per cent)	80_1	0.0955	89_2	0.0482	0.047

The increase of opacity of the blood-water mixture after exercise is associated with reduced pH values of the plasma. When blood is equilibrated in a tonometer with different concentrations of CO_2 the opacity increases with the concentration of CO_2. The same is true when small amounts of lactic acid and of HCL are added to the blood. Alkalinization of the blood results in reduced degrees of opacity. When CO_2 or the breath is blown into the water before the blood is added to it, or when the pH of the water is reduced, for example, by addition of small amounts of KH_2PO_4 or of acetic acid, the opacity of the blood-water mixture is increased to a degree roughly parallel to the acidity of the water.

It must be emphasized that the resistance of a small percentage of the red blood cells to laking by water is not necessarily related to the total span of resistance of the majority of red blood cells to hypotonic salt solutions. Indeed, in vitro experiments show that rendering the blood more acid *reduces* the resistance of the *majority* of red blood cells to hypotonic salt solutions. This has been shown previously by Ham (3). It is less clear what happens to the bulk of the red blood cells in this respect in man after exercise. Hastings found that in dogs after exercise the resistance of the red blood cells was increased (4).

Chemical changes of the blood during strenuous exercise influence in some manner the resistance of the red blood cells to laking. Thus, a rough parallelism has been shown to exist between the opacity of the blood-water mixture and both the blood lactic acid and the degree of exhaustion after a run. This is illustrated in tables 1 and 2. When the speed of the treadmill was increased the opacity values increased in every case. When the blood lactic acid was about 50 mgm. per 100 cc. the opacity range was approximately from 0.024 to 0.035. When the lactic acid was about 100 mgm., the opacity range was approximately 0.028 to 0.045. When the lactic acid was about 140 mgm. the opacity range was approximately from 0.045 to 0.072. There was an inverse relation between the physical fitness index and both the lactic acid and the opacity reading. In the untrained men, the opacity increased with the duration of the run and was greater in younger individuals.

Other changes of the blood in addition to the lactic acid undoubtedly influence the resistance of the red blood cells. In the untrained men the opacity on the whole was less for given values of lactic acid than in the trained men. In another series of eight individuals exercising on the treadmill there was close relation between the maximum pulse during exercise (taken with a cardiotachometer) and the opacity of the blood taken 5 minutes after exercise, whereas the lactic acid values did not show a relation to maximum pulse in this group of subjects. In the larger series reported here, both opacity and lactic acid

were roughly related to the pulse rate one minute *after* exercise, the lactic acid-pulse relation being the closer.

TABLE 1

Blood "opacity" readings and other data in college cross country runners (ages 17–21)

Blood samples were taken 5 minutes after a run on a motor-driven treadmill. Cases are arranged in the first part of the table according to the degree of "opacity." In the second part of the table are shown the results obtained on the same subjects after a faster run.

CASE NO.	TREADMILL SPEED: 7 M.P.H., GRADE 8.6%				TREADMILL SPEED: 8.6 M.P.H., GRADE 8.6%			
	Duration of run	Physical fitness index*	Lactic acid	"Opacity" ($L_1 - L_2$)	Duration of run	Physical fitness index*	Lactic acid	"Opacity" ($L_1 - L_2$)
	seconds		*mgm./100 cc.*		*seconds*		*mgm./100 cc.*	
1	300	99	48	0.024	300	106	113	0.043
2	300	106	26	0.026	300	112	108	0.044
3	300	91	28	0.028	300	97	96	0.040
4	300	98	50	0.028	300	103	116	0.043
5	300	80	48	0.030	300	116	89	0.043
6	300	96	48	0.030				
7	300	78	79	0.030	192	64		0.064
8	300	86	50	0.031	300	107	119	0.049
9	300	85	49	0.034	300	97	169	0.075
10	300	92	50	0.034	300	98	119	0.050
11	300	95	52	0.034				
12	300	85	85	0.037	167	58	121	0.065
13	300	88	60	0.037				
14	300	83	65	0.038	215	76	113	0.049
15	300	75	99	0.042	195	57	180	0.079
16	300	79	107	0.042				
17	300	82	92	0.043	217	68	118	0.055
18	300	81	124	0.048				
19					274	88	126	0.062
20					300	101	146	0.065
21					265	73	157	0.065
22					300	98	145	0.073
Average...	300	87.7	64.4	0.0342	266	89.3	127.1	0.0567

* The physical fitness index is obtained by dividing the duration of the run in seconds ($\times$ 100) by twice the sum of pulses 1–1½, 2–2½ and 4–4½ minutes after the run (5). In the second part of the table, physical fitness index was multiplied by 1.26 in order to make the index comparable to that obtained when the speed of the treadmill was 7 miles per hour.

The test for opacity as described has proved in this laboratory to be a simple and brief method for judging the degree of exhaustion and in a manner the determination and grit of young men placed in conditions requiring severe exertion. It will assist in judging the physical fitness of men. Further study of the factors of the blood in exercise, responsible for the changes in resistance

of red blood cells, should add to knowledge of the physico-chemical equilibria of the body.

TABLE 2

Blood "opacity" readings and other data in miscellaneous group of men, out of condition (ages 25–57)

Blood samples were taken 5 minutes after a run on a motor-driven treadmill (7 m. per hr., grade 8.6%). Cases are arranged according to degree of "opacity."

CASE NO.	AGES	DURATION OF RUN	PHYSICAL FITNESS INDEX*	LACTIC ACID	"OPACITY" ($L_1 - L_2$)
		seconds		*mgm./100 cc.*	
23	57	62	18	83	0.026
24	40	82	26	95	0.028
25	40	92	25	106	0.029
26	38	85	23	88	0.033
27	30	75	18	100	0.033
28	37	67	17	105	0.033
29	35	89	25	111	0.033
30	45	85	23	112	0.033
31	32	63	18	62	0.035
32	48	77	24	103	0.036
33	31	67	16	113	0.038
34	31	73	18	103	0.039
35	33	76	18	117	0.041
36	26	94	24	131	0.041
37	35	53	13	93	0.042
38	42	68	18	87	0.043
39	32	77	17	128	0.043
40	30	122	28	129	0.043
41	43	70	17	117	0.044
42	34	22	5		0.045
43	34	145	39	133	0.047
44	42	135	30	137	0.047
45	38	87	18	138	0.047
46	31	155	42	108	0.049
47	25	185	47	157	0.053
48	26	100	24	133	0.056
49	28	163	40	135	0.056
Average	35	91.4	23.4	112.4	0.0404

* See footnote to table 1.

SUMMARY

A small percentage of circulating red blood cells is resistant to laking by water. The opacity which results when a small amount of blood is added to a large amount of water can be measured by a simple method employing the photoelectric colorimeter. This opacity increases after exercise, is dependent on the severity of the exercise, and is roughly proportional to the lactic acid of the blood. The opacity increases when blood is exposed in vitro to various

acids. Undoubtedly factors in addition to the lactic acid concentration which change in vivo with exercise, influence the resistance of the red blood cells.

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THE VASOPRESSOR EFFECT OF THERMAL TRAUMA[1]

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In recent experiments on gastric secretion and motility in burns (1) it was observed that the blood pressure frequently rose during the burn and often remained for some time at or above the control level. This was contrary to our experience with the changes in blood pressure following traumatization (2). A review of the literature revealed that, although many others had reported similar observations, no extensive analysis of this phenomenon has been made. A preliminary report on the causes of the elevation of blood pressure following burns has been presented previously (3), and this paper is the complete report and an extension of the work.

Simultaneously with our report (3), Kabat and Hedin attributed the rise in blood pressure during burns to a nervous factor (4, 5). They found that, following thermal trauma to normal anesthetized cats, an immediate and sustained rise in blood pressure occurred, while in similar experiments with spinal cats a temporary fall in blood pressure was followed by a lesser rise. Similar observations had been made by others. In 1901, in a monograph on his extensive work, Crile (6) described the effect on blood pressure of a nervous factor in burns. He prevented the rise of blood pressure following burns of the hindlegs of cats by injecting cocain into the subarachnoid space of the lumbar spinal cord or by blocking the afferent nerves. In 1931 a rise in blood pressure following burns in dogs was reported by Johnson and Blalock (7) who stated: "During the time that heat was being applied, the blood pressure usually fell slightly. This was not always the case as in some instances it definitely rose." Harkins (8), in 1935, reported that in two of three burn experiments in dogs a rise in blood pressure occurred, and in his monograph (9) he cited similar results from a number of authors. In a detailed study of burns, Lambret and Driessens (11) in 1937 performed experiments on spinal cats similar to those of Kabat and Hedin (4, 5). They believe that the rise in blood pressure during the burn was due to a sudden discharge of epinephrin. This view is supported by the earlier findings of Hartman et al. (12) in 1926, that the concentration of epinephrin in the blood of cats is increased following burns. The rise in blood pressure after burns was also reported in humans by Wilson (10) in 1938. He states: "Forty cases—were admitted within 2 hours after injury; in 35, and amongst these were some of the most extensive and severe injuries, the systolic level of blood-pressure was normal or, in a few, actually above normal."

Our preliminary work (3) convinced us that the pressor effect of burns was

[1] This work was done under the auspices of the Committee on Research in Shock, of the Michael Reese Hospital, and was supported by the Michael Reese Research Foundation and by the Gusta M. Rothschild Fund.

not due to any single factor. We, therefore, have performed 125 experiments on dogs and cats in an attempt to analyze this phenomenon further.

EXPERIMENTAL PROCEDURE. Normal, healthy, fasting dogs and cats were used. All animals were kept under deep anesthesia with sodium pentobarbital (nembutal), diallylbarbituric acid (dial), sodium isoamylethybarbiturate (sodium amytal), sodium barbital and morphine or ether. Only acute experiments were performed, at the termination of which an excess of anesthetic was administered. Blood pressure was recorded from the carotid artery with a mercury manometer. Third degree burns were administered with boiling water or with a torch. The duration and extent of the burns varied in different experiments. Circulating time was determined by the sodium cyanide method as described previously (13). Hematocrit values were obtained with Wintrobe tubes centrifuged at 2000 r.p.m. for 30 minutes. Arterial plasma CO_2 was determined by the manometric method of Van Slyke and Neill and plasma proteins by the falling drop method (14).

TABLE 1

Effect of burns on blood pressure

GROUP NO.	SPECIES	TOTAL NO.	PROCEDURE	AVERAGE BLOOD PRESS. CHANGE ±
				mm. *Hg*
1	Dog	26	Scalds or burns	+50
2	Dog	2	Burns, const. inj. A.C.*	+75
3	Dog	4	Hypophysis exposed, burn	+50
4	Dog	3	Hypophysectomy, burn	−45
5	Cat	15	Foot scalded	+60
6	Cat	7	Denervated foot scalded	−20

* Acetylcholine iodide, constant intravenous injection.

RESULTS. In table 1 six groups of experiments are summarized. Following extensive torch burns or scalding, twenty-six dogs (group 1) showed an average rise in blood pressure of 50 mm. Hg. Figure 1 represents one typical experiment; the columns of data demonstrate blood changes. The data in the left column are controls, in the middle column, one hour after the first scald, and in the right column, one hour after a second scald. The immediate result of the first (severe) scald was a sharp rise of the blood pressure which remained elevated for 15 minutes. One hour after the burn, the blood pressure was at approximately the control level. The effect of the second scald on blood pressure was slight. One hour later the blood pressure still had remained at a normal level, but the animal was in shock, as indicated by the other determinations. Considerable hemoconcentration, acidosis and slow circulation were present, and the animal died shortly. Six similar experiments with blood studies were performed; the average changes one hour after a single scald were as follows: a slight rise in blood pressure, a 10 second increase in the circulating time, a hemoconcentration of 24 per cent, and a 13 volume per cent in arterial plasma CO_2.

The above studies were continued on the cat which seemed to give a greater pressor response to burns than the dog (group 5, table 1). After some experimentation as to extent, type and degree of burn necessary to give fairly constant results, we found that immersing one foot up to the ankle in boiling water for 30 seconds gave the most consistent results. The effect was an immediate sharp rise of blood pressure, which was maintained in many experiments for a considerable period of time. As shown in table 1, group 5, the average rise of blood pressure in 16 cats was 60 mm. Hg. In a typical experiment, represented in figure 2, the blood pressure was still elevated 35 mm. Hg above the control level fifty minutes after scalding. At that time, the hematocrit value had increased from 36 to 47 per cent indicating a peripheral red cell concentration of 31 per cent above the control level. One and one-half hours after the burn, the blood

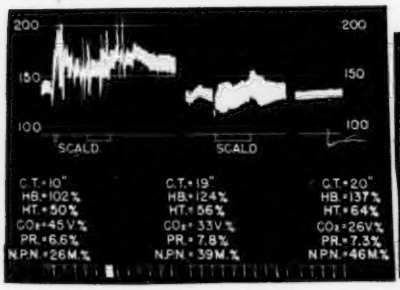

Fig. 1

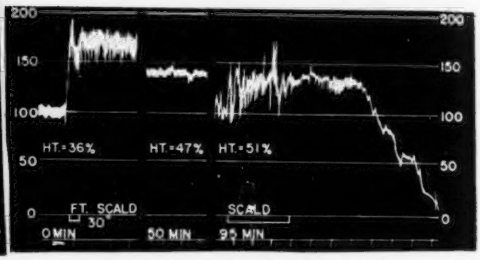

Fig. 2

Fig. 1. Normal dog, male, 18 kgm. Nembutal. Blood pressure tracing during and after a severe scald. Time in minutes. *C.T.* = circulating time; *HB.* = hemoglobin; *HT.* = hematocrit; CO_2 = arterial plasma CO_2; *PR.* = plasma proteins; *N.P.N.* = non protein nitrogen.

Fig. 2. Normal cat, female, 3 kgm. Nembutal. Blood pressure tracing during and after scalding.

pressure had returned to the control level. However, seven minutes after an additional scald this animal died, although the blood pressure was well maintained until a few minutes before death.

In order to explore the possibility of a nervous factor in burn shock, experiments were performed on cats in which one leg was denervated by section of the sciatic and femoral nerves. The central end of the cut sciatic nerve was stimulated with faradic current to simulate an increase in the number of afferent impulses from a burned area. Figure 3 represents a typical experiment. When the nerve was stimulated with alternating induced current (first arrow, fig. 3) there occurred a sharp rise in the blood pressure similar to that observed after a burn. With the cessation of the stimulation (second arrow) the blood pressure immediately dropped to slightly below the control level. This response differed somewhat from the usual response to a first burn, but after a burn pain impulses may continue to arise for some time. After the blood pressure had returned to the control level, the denervated foot was scalded for 30 seconds, and the blood pressure did not rise during the burn but dropped slightly.

After a short interval, the intact foot was scalded. The blood pressure rise was marked but only of short duration. This transitory rise in blood pressure occurred in many experiments; that is, a second burn rarely would have as striking a result as the first one. The pressor phenomenon was more constant and was usually greater and of longer duration with the first burn. The depression of blood pressure, following the scalding of a denervated limb, was confirmed in seven experiments, in which the average drop of blood pressure was 20 mm. Hg. These experiments are listed in group 6 of table 1.

The abolition of the typical pressor response by deafferentation of the limb before scalding suggested a reflex mechanism, possibly through the splanchnic nerves. Therefore, a series of experiments was performed on acutely adrenalectomized cats and on splanchnectomized dogs. Only such animals were used, which had a more or less normal blood pressure following the operation. The

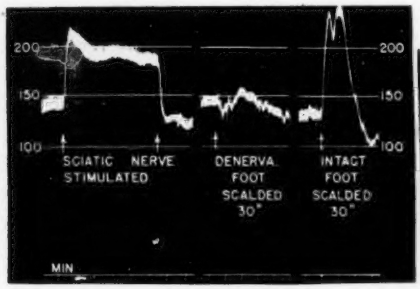

Fig. 3

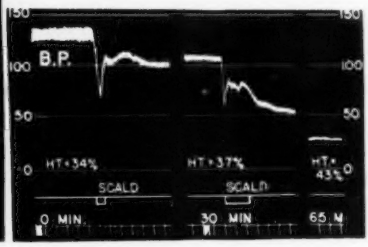

Fig. 4

Fig. 3. Cat, female, 4 kgm., denervated leg. Nembutal. Blood pressure tracing; fremoral and sciatic nerves sectioned on one side.

Fig. 4. Dog, male, 16 kgm., hypophysectomized. Nembutal. Blood pressure tracing.

blood pressure rose immediately when the animals were burned, but in the adrenalectomy experiments the initial rise was less and the duration of the rise was shorter than in the controls.

The role of the spleen as a significant factor in the vasopressor effect of burns was investigated on acutely splenectomized dogs. The vasopressor response to a scald was not abolished by splenectomy.

The experiments on adrenalectomized and splanchnectomized animals had shown that the adrenal medulla and the splanchnic nerves played only a minor rôle if any in the reflex rise of blood pressure following burns. Therefore other mechanisms had to be considered. Lim and associates (15) and Necheles and Neuwelt (16) have shown that pituitary pressor hormone can be liberated reflexly by stimulation of afferent nerves. Therefore the next series of experiments were performed on dogs, hyphophysectomized by the temporal approach. One hour after the operation, the blood pressure of the animals usually had returned to near control levels and the first scald was administered; in all three experiments performed, blood pressure dropped immediately. The average drop in blood pressure was 45 mm. Hg (table 1, group 4). This drop was similar to that

seen following mechanical traumatization (17). Four control experiments (table 1, group 3) were done in which the hypophysis was exposed, but not removed. Blood pressures in these sham experiments were at or near control levels at the time of the burn, and the pressor response to a burn was similar to that of the normal animal (table 1, group 1).

The experiments with exclusion of the hypophysis seemed to prove that the reflex rise of blood pressure following a burn was of a neuro-humoral nature. However, the absence of the secretion of a pressor hormone of this gland might have changed capillary tone in such a way that we were working with a different biological test object. In order to avoid this objection, we attempted to detect an increase of pituitary pressor hormone following burns in dogs with intact hypophyses.

In 1938 Necheles and Neuwelt (16) described a sensitive biologic test for pitressin. They found that small doses of pitressin or of pituitrin injected intravenously, inhibited the vasodepressor effects of small doses of acetylcholine and that stimulation of the central end of the vagus nerve was followed by inhibition of the vasodepressor effect of acetylcholine only in the presence of the hypophysis. Thus, if pituitrin was liberated during a burn, small doses of acetylcholine should have no effect on the blood pressure. In the four experiments performed, normal vasodepressor responses to acetylcholine were seen before, but none occurred after a severe burn. In 2 dogs, in which reflex liberation of pituitary pressor hormone had been precluded by hypophysectomy, no change in the acethylcholine response after a severe burn was observed. In both animals a drop of blood pressure followed the burn.

Following burns in normal dogs the acetylcholine response disappeared and, in severely burned animals, did not return to the control level. In three experiments a normal response to acetylcholine was present after a mild burn although a rise in blood pressure had occurred.

Since the above experiments pointed to the hypophysis as one important cause of the rise of blood pressure following burns, the next logical step in our experiments seemed to be the transfusion of blood from burned animals into assay animals and the testing of the latter for the presence of pituitary pressor hormone with injections of acetylcholine, as described above.

The transfusion of large amounts of blood from burned into normal assay animals suppressed the vasodepressor effects of acetylcholine. Figure 5 represents one typical experiment. Control injections of graded small amounts of acetylcholine produced typical, sharp, transient depressions of blood pressure. After this response had been found to be constant, 250 cc. of fresh heparinized blood from a burned dog was injected intravenously. The acetylcholine response diminished gradually and, 1½ hours after the injection, it was completely absent. This rather slow disappearance of the depressor effects of acetylcholine was entirely different from the effect of pituitrin or pitressin on the typical acetylcholine response (16). In the latter case, inhibition of the acetylcholine response was present shortly after the injection of pituitrin, and it gradually returned to the control response.

The above experiments made us feel that although the pituitary pressor hor-

mone might play a rôle in producing a rise of blood pressure following burns, it could not explain the entire phenomenon. The next question which presented

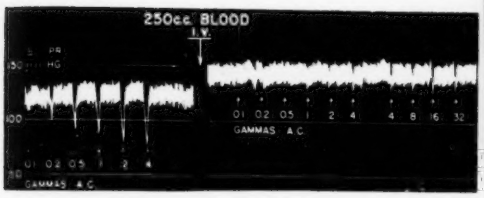

Fig. 5

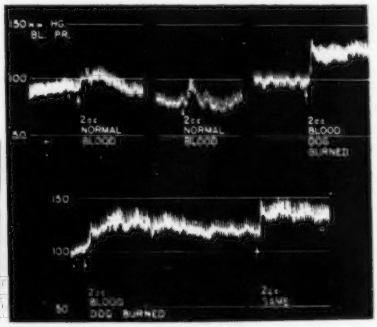

Fig. 6

Fig. 5. Normal assay dog. Nembutal. Response to A.C. following the injection of blood from a burned dog. At the left side of the record, graded small amounts of acetylcholine iodide produced typical effects. The right part of the record, taken 90 minutes after injection of blood from a burned dog, shows that the effects of acetylcholine were absent.

Fig. 6. Eserinized cat, 3 kgm. Dial. Response of blood pressure to injection of blood from a burned dog.

TABLE 2

Injection of blood from burned animals

EXPT. NO.	BURNED ANIMAL	ASSAY ANIMAL		DRUGS
		Species	Blood press. effect	
			mm. Hg	
PR 1	Dog	Cat	+45	Eserine
PR 2	Dog	Cat	+25	Eserine
PR 4	Dog	Dog	+15	
PR 5	Dog	Dog	+15	
PR 10	Cat	Cat	+20	
PR 12	Dog	Dog	+35	A.C. const. inj.*
PR 13	Dog	Dog	+15	
PR 20	Dog	Dog	+65†	
PR 23	Dog	Dog	+15	A.C. const. inj.
PR 3	Cat	Dog	0	
PR 9	Dog	Dog	0	
PR 10	Cat	Cat	0	
PR 13	Dog	Dog	0	
PR 24	Dog	Dog	0	

* Acetylcholine iodide, constant intravenous injection.

† Large infusion.

itself was whether blood from a burned animal would affect the blood pressure of a normal assay animal.

The upper part of table 2 presents a summary of the experiments dealing with the effect of injections of blood from burned animals into assay animals. In 9

out of 14 experiments a distinct rise in blood pressure from +15 to +65 mm. Hg resulted. The pressor responses were accentuated when the assay animals had been made hypotensive (about 100 mm. Hg mean pressure) with eserine or acetylcholine. Figure 6 represents a typical experiment in which a pressor response was obtained with small quantities of blood (2 cc.); normal blood from the test animal before the burn had only a slight and transitory effect, while the injection of blood drawn after the burn produced a considerable pressor effect, a rise of 25 mm. of blood pressure.

Following burns in dogs, cats and rabbits, an outstanding observation was the immediate appearance of free hemoglobin in the plasma (the question of muscle hemoglobin should be investigated), the amount depending on the severity of the burn. Therefore we had to consider whether normal partly hemolyzed blood per se would affect the blood pressure of an assay animal. The results showed that slightly hemolyzed normal blood, injected intravenously into a normal dog, caused a pressor response. These last findings seem to be important, because one of our most consistent findings has been the presence of hemoglobin in the plasma of burned animals.

Hemolysis in vivo is known to be able to affect kidney secretion. During and following burns, kidney secretion was depressed in all animals, the degree of the depression depending on the extent of the burn. The kidneys of these animals were of chocolate brown colour and were found to be engorged with hemoglobin. It was conceivable, therefore, that an acute hypoxia of the kidneys might follow a burn, and that consequently large amounts of renin would be produced, which would raise the blood pressure. Therefore, acutely nephrectomized animals were subjected to scalding. The typical vasopressor response of burns was found in these experiments.

Discussion and conclusions. We have shown, in confirmation of earlier work, that a reflex mechanism is present in burns, which maintains a more or less normal or even an elevated blood pressure for a considerable length of time. This relatively normal or elevated blood pressure probably explains the rarity of "primary" or "initial" shock (immediate low blood pressure, 10) in humans and animals suffering from severe burns. Wilson (10) in reporting a series of cases of very severe burns stated: "initial shock was infrequent, usually of moderate severity and transient. Profound initial shock was associated with unusual circumstances." However, this relatively normal or even elevated blood pressure gives no indication of the true condition of the animal, for the other experimental findings reveal hemoconcentration, acidosis and increased circulating time. Therefore, if the blood pressure had been the only criterion considered the animal's condition would have been called excellent. The blood pressure can be within normal limits until a few minutes before death. Hematocrit, circulating time and plasma CO_2 values are more reliable indices of damage suffered after thermal trauma. Kabat and Hedin (4, 5) in subacute experiments found slightly longer survival times in spinal animals subjected to a burn than in the case of normal animals. They apparently did not employ a standard degree of thermal trauma; the wide range of the survival times would seem to necessitate a larger series of experiments than employed by them.

We feel that the vasopressor effect of burns had a beneficial effect in the case of our acute experiments; survival times were longer than in comparable experiments with mechanical traumatization, and circulating times were shorter following burns than following mechanical traumatization (13). We feel justified to conclude therefore that, at least in the early stages of burn shock, the maintenance of a more or less normal or of an elevated blood pressure may be beneficial to the organism.

The spleen was excluded as an important factor in the vasopressor effect of burns. This was surprising, because in the dog this organ may contain and expel large amounts of blood.

In an analysis of the reflex mechanisms of the rise of blood pressure in burns, the adrenal medulla and the splanchnic nerves were found not to play an important rôle. Bilateral adrenalectomy modified the curve of the blood pressure rise following a burn, somewhat, but did not abolish it.

The absence, after hypophysectomy, of the typical pressor response following burns does not prove that the hypophysis plays a rôle in the maintenance or rise of blood pressure following a burn, because the organism has been deprived of the tonus hormone of the capillaries; in other words, the reactions of the test animal have been altered. The change in the biologic test with acetylcholine for pituitary pressor hormone was startling, but in no way typical for pituitary pressor hormone. Yet, we cannot dismiss the idea that the considerable degree of hemolysis present in every severe burn could not modify the vasomotor response to acetylcholine to a slowly developing and prolonged reaction.

Our results with normal blood hemolyzed in vitro and with hemolyzed blood from burned animals indicate that, besides the nervous or reflex factor, a physical factor may play a rôle in the pressor phenomenon of burns. In burned animals, kidney secretion slowed down immediately after severe burns and often stopped completely. The kidneys were found engorged with hemoglobin. It was, therefore, conceivable that due to an early hypoxia of the kidneys, renin may enter the circulation. The rôle of renin as a major factor in the vasopressor effect of burns was excluded, however. After having excluded the rôle of the adrenals, the splanchnic nerves, the spleen, and the kidneys as important factors in the vasopressor effects of burns, the following factors remained: hemolyzed blood, pain impulses from the burned area, and the posterior pituitary gland. While the evidence for the first two factors is good, the rôle of the pituitary pressor hormone is not proven, but cannot be ruled out.

SUMMARY

1. During and following burns a rise of blood pressure occurred, which in many experiments remained elevated for a considerable period of time following the burn.

2. This rise in blood pressure may give misleading information concerning the condition of the animal.

3. Scalding of a denervated foot caused a slight drop in blood pressure; scalding of the normal foot resulted in a marked rise in blood pressure.

4. Splenectomy did not abolish the pressor effect of burns.

5. Adrenalectomy, or splanchnectomy, did not abolish the pressor effect of a burn.

6. Hypophysectomized animals exhibited a marked fall in blood pressure when burned, the opposite of the response of normal animals.

7. The acetylcholine vasodepressor response was inhibited in severe burns.

8. A pressor substance could be demonstrated in blood from burned animals, but part of its effect seemed to be due to hemolysis.

9. Renin was excluded as a major factor in the vasopressor effect of burns.

10. The rise of blood pressure following burns seems to be due to a combination of several factors, nervous, hormonal and hemolyzed blood.

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CARDIOVASCULAR ADJUSTMENTS OF MAN IN REST AND WORK DURING EXPOSURE TO DRY HEAT[1]

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It is well recognized that men accustomed to high temperatures can perform fixed physical tasks in the heat with better regulation of body temperature than unacclimatized men. For example, Dill (1-A) demonstrated that a stay of three weeks in the desert produces a marked improvement in the body temperature response to work in the heat. However, there is little evidence as to the rate of acclimatization to the heat or as to the detailed mechanisms involved. All indications are that cardiovascular adjustments are predominant. The cardiovascular studies of Scott et al. (2) were limited to mild heat without physical exertion.

The questions which we have emphasized are: 1. How fast do men acclimatize to heat during the first few days of exposure? 2. How great is the variation between individuals? 3. Can we predict which men will respond best to heat? 4. What is the sequence of the adjustments involved in establishing acclimatization to heat?

These questions have been studied in normal men under rigidly standardized and controlled conditions. This is a report on the cardiovascular findings and related subjects. The principal data comprise some 7,000 observations made on 43 subjects in the course of 202 subject days. Occasional reference is made to results on 23 additional subjects studied during 147 subject days.

Subjects. The subjects used in these experiments were volunteer soldiers from the 710th M. P. Battalion and hired men students of the University of Minnesota. All subjects were free from major physical defects which might affect cardiovascular functions. Ages varied from 18 to 46, most of the men being in the 20- to 30-year age group.

General procedure. Experiments of 3 different durations were used. In the initial series 6 men were studied in an 8-day exposure to the heat. These experiments, together with other evidence, indicated that the major cardiovascular adjustments are completed in about 4 days and that the first 2 days of exposure are critical. Accordingly, a second series was carried out in which 12 men were studied during 3½ days exposure (series II) and 25 men were studied during 2 days' exposure (series III). In all cases the men were also studied under temperate conditions (control) for 2 days immediately prior to the hot periods.

[1] The work described in this paper was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Minnesota. Acknowledgment is also made of support from the Nutrition Foundation, Incorporated.

During the hot periods the subjects ate, slept and worked continuously in the controlled condition suite.

A standard fixed regime of diet, observations, physical work, special tests and rest was followed. Standard clothing consisted of cotton shorts, socks and shoes for work and, for evening and rest periods, standard U. S. Army one-piece Armored Forces battle dress. Physical work consisted of marching, in groups of 2 to 4 at a time, on a large motor-driven treadmill. The rate was constant at 3.25 miles per hour at an angle of climb of 7.5 per cent. This work demands oxygen consumption at about 7 times the basal rate. The work regime alternated work and rest in periods of 10 minutes each.

TABLE 1

Protocol for daily routine

TIME	TEMPERATURE	ACTIVITY	OBSERVATIONS
	°F.		
7:30– 8:00 a.m.	85	Basal	Rectal temperature Pulse, weight Crampton test
8:00– 8:30 a.m.	95	Breakfast	
8:30– 9:15 a.m.	105	Rest	Cold pressor test
9:15–12:00 a.m.	110–120	8 work-rest periods, 20 min. each	Work pulse rate Rectal temperature Rate of sweating
12:00–12:30 p.m.	110*	Lunch	
1:30– 4:15 p.m.	120	8 work-rest periods, 20 min. each	Work pulse rate Rectal temperature Rate of sweating
4:35– 5:10 p.m.	120	Rest	Crampton test Cold pressor test
5:10– 6:00 p.m.	110	Rest	
6:00 p.m.	90	Supper	
6:00– 7:30 a.m.	85		

* A 10°F. difference between dining room and treadmill room.

Particular attention was paid to pulse rates in rest and work, rectal temperature, rate of sweating, cardiovascular postural adjustment tests and cold pressor tests. An outline protocol of procedure for a typical day is given in table 1.

Environmental Conditions. The air temperature in the hot periods was 120° F. dry bulb and 85° wet bulb during the day and 85° dry bulb and 65° wet bulb at night. The "effective" temperatures (Houghton and Yaglou, 3) were 92° and 74°, respectively. The control period temperature was the same as at night during the hot periods. The transition from night to day conditions required $2\frac{1}{2}$ hours in the morning; 110°F. was reached within the first hour. The evening transition was completed more rapidly. Air movement was constant at all times. Radiation exchanges were not completely controlled; the wall temperature slowly followed the air temperature, reaching 118°F. in the late afternoons.

METHODS. *Pulse rates* were counted with the aid of a stethoscope in rest and at the end of the work periods on the treadmill. The latter count was begun within one second of cessation of work and continued for 15 seconds. The a.m. and p.m. "work pulse rates" referred to subsequently in this report are the averages of 8 measurements for each man.

Body temperature was measured rectally with a clinical thermometer. Rectal temperatures were measured during the first 90 seconds of recovery after the second, fourth, sixth and eighth work periods of each half day.

Postural circulatory adjustment was studied by means of the "blood ptosis" test of Crampton (4). The subject reclined horizontally on a tilt table until 2 pulse counts agreed within one beat per minute; the blood pressure was then measured. The table was then tilted to an angle of 65° and the pulse rate counted until stabilization was reached as before; the blood pressure was measured at this moment. The original Crampton test score does not provide for changes as large as observed in the heat so a modified scoring system was devised which extends the range.

The "cold pressor" test of Hines and Brown (5) was carried out with measurements of blood pressure and pulse rate before, during and after a 60-second immersion of the left hand in ice water.

Rate of sweating was measured by weight loss of the nude subject after careful drying. The balance used was calibrated to $\frac{1}{4}$ ounce. Rate of sweating was measured over the third work and the succeeding recovery period in the morning and again over the sixth period in the afternoon. The 24-hour sweat volume was calculated according to the formula: sweat loss equals water drunk plus water in food plus water of metabolism minus the sum of urine water plus fecal water plus water lost through the lungs. Water drunk, urine volume and weight of food were measured; the other variables were estimated. The final value was corrected for the 24-hour weight change.

Diet and water. A constant diet was provided by a trained dietitian. It contained 3,100 calories, 95 grams of protein and 120 grams of fat. The diet provided 15 ± 2 grams of salt and was adequate, according to ordinary standards, in vitamins. On several experiments the salt intake was reduced to 5.8 ± 2 grams. Water was allowed *ad lib* at all times except during observations and actual work on the treadmill.

RESULTS IN WORK. Pulse rates and rectal temperature responses to work during the 8 days of exposure to heat are given in figure 1. Pulse rates for eight work periods for each man for each half day were averaged and the means for six men appear as a single point in the figure. Similarly, the rectal temperature points in the figure each represent the average of 48 determinations on six men for each half-day period. The pulse rates and rectal temperatures showed marked progressive acclimatization; after four days in the heat, the work was done in the heat with a pulse rate and rectal temperature not far different from that for work in the cold (control). There was little or no change from the 5th to the 8th day in the pulse rate or rectal temperature.

The rate of sweating in work (p.m. values) increased 26 per cent over the 8-day period. However one-half the pulse rate and rectal temperature changes were

completed on the 3rd day with only a 4 per cent increase in the rate of sweating, and one-half of the total increase in the rate of sweating during work took place after all adjustments in temperature regulation as measured by rectal temperatures had taken place.

All these changes took place without marked dehydration. Mean weight changes in grams during the day are given in table 2 along with rates of sweating during work and total 24-hour sweat volumes.

RESULTS IN REST. Results in rest are given for series II since the most striking changes occur during the first 3½ days. Values for pulse rate and blood pressure

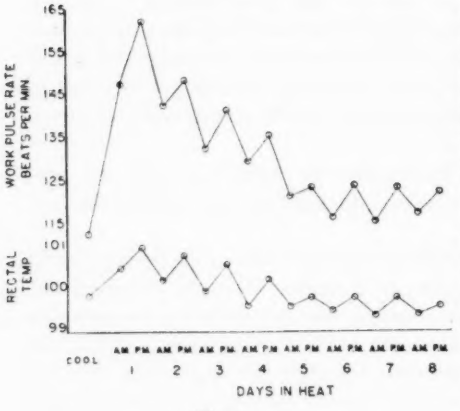

Fig. 1

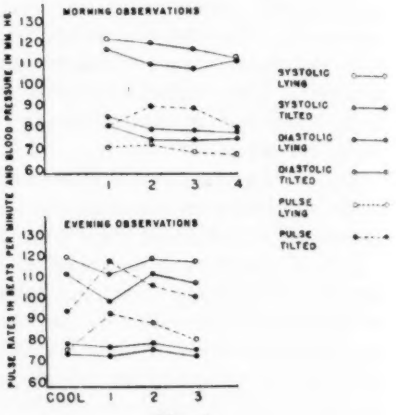

Fig. 2

Fig. 1. The mean work pulse rates and rectal temperatures of six men exposed to eight days of heat.

Fig. 2. The pulse rate and blood pressures of 12 men (series II) during a 3½ day exposure to heat. The upper chart represents basal observations in the morning (85°F.); the lower, observations 20 minutes after work. Note that morning observations on first day of heat serve as a control.

before and after elevation of the tilt table are given in figure 2. Values for 12 men were averaged for morning tests and for afternoon tests.

Marked changes were produced by the heat. In general these alterations tend to return to normal values by the second or third day of exposure to the heat, but the morning systolic blood pressure in the horizontal position seems to be an exception.

Indices of cardiovascular efficiency were calculated by the modified Crampton method (see "Methods"). The average results are listed in table 3; the statistical significance is analyzed in table 4.

Cold pressor test. Information on the state of the autonomic nervous system before, during and after exposure to heat might be expected to be of value, since cardiovascular adjustments to heat depend to a great extent on the proper distribution of the circulating blood. The results of cold pressor tests performed on

15 men in series 3 are summarized in figure 3. Each subject was given 4 trial tests before exposure to heat.

Analysis in terms of Fischer's t values showed the only significant difference occurred between the blood pressure before and during stimulation with ice water in the morning experiment after one day in the heat. A few individual differences were noted. In the 15 subjects studied, two could be classified according to Hines

TABLE 2

The rate of sweating in grams per minute during work, the total 24-hour sweat loss, the weight changes between the 2nd period in the morning and the 6th period in the afternoon, mean body weight, and the 24-hour urine volume

	DAYS IN HEAT				
	1	3	5	7	8
Rate of sweating (gms./min.)					
a.m.	13.3	13.8		14.5	14.1
p.m.	13.4	13.9	15.2	16.7	16.9
24-hour sweat volume (liters)	6.628	6.602	6.468	6.997	6.786
Mean change in wgt. from morning to afternoon* (gms.)	+187	+172	−24	0	−12
Mean body wgt., a.m., (lbs.)	152.8	152.3	152.0	154.4	153.2
24-hour urine vol.	750	809	1,124	919	1,083

* To the 6th period in the afternoon.

TABLE 3

Average Crampton scores (modified) for successive days

	CONTROL DAY	1ST DAY HEAT	2ND DAY HEAT	3RD DAY HEAT	4TH DAY HEAT
Morning		71	61	60	72
Afternoon	60	46	61	56	

TABLE 4

Statistical analysis of cardiovascular fitness indices from table 3

COMPARISONS	DEGREES OF FREEDOM	t VALUE OBTAINED	t VALUE AT 1% LEVEL
Control p.m. vs. 1st heat p.m.	11	5.88	3.10
First heat a.m. vs. 2nd heat a.m.	11	3.51	3.10

and Brown (3) in the normal hyper-reactive group before exposure to heat. After one day in the heat, four new subjects appeared in this group and one dropped out.

Individual variation and interpretation. The average results indicate that adaptation to high temperatures is well started by the second day. However this is not true of a significant number of subjects. Table 5 gives the per cent of men studied from the three series who did not show improvement after the first day as judged by work pulse, rectal temperatures and Crampton score.

Nine men failed to show improvement in the work pulse rate on the second day. Seven of these men had no improvement in rectal temperature and only two showed definite improvement in the Crampton score. Thus 13 per cent of the subjects on the second day had definitely not improved by all the criteria used. On the fourth day one man who failed to show any improvement by any of the criteria used had lost 4.5 per cent of his weight and had undoubtedly handicapped himself by failing to drink sufficient water for his needs. Dehydration was also present in the three other men who failed to show improvement in either the Crampton score or the rectal temperature.

Ten men out of 66 studied in this laboratory suffered heat exhaustion requiring interruption in the work schedule. Four of these exhibited clear cut cases of

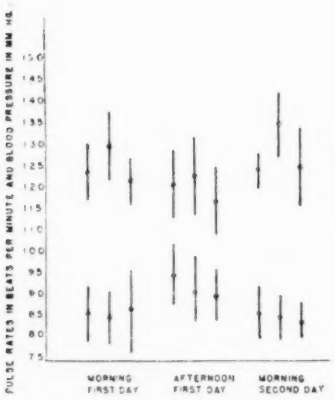

Fig. 3

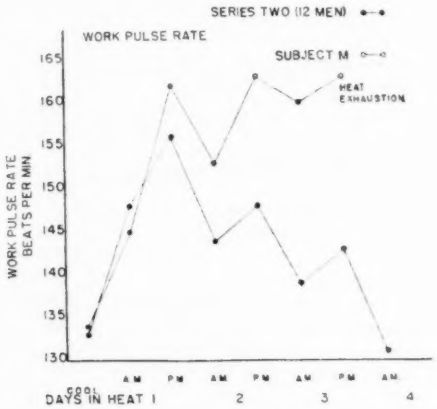

Fig. 4

Fig. 3. Pulse rates, blood pressures and their respective standard deviations of a group of 15 men before, during and after exposure of the hand to ice water for one minute.

Fig. 4. The work pulse rate of subject M who suffered heat exhaustion characterized by hypotension, tachycardia, vertigo and vomiting on the afternoon of the third day in the heat compared to a group of 12 men (series II) who acclimatized in a normal manner.

heat prostration characterized by low resting blood pressure, rapid pulse, nausea, vomiting and vertigo. Three of these cases occurred on the second day in the heat; one on the third day. Figure 4 shows the failure of subject M to have any improvement in his work pulse rate until he finally suffered heat prostration on the afternoon of the third day. The work pulse rates of the other three men who suffered heat exhaustion on the second day were either higher than or equal to the values of the first day. Table 6 gives the average of findings on these four men on the evening of the first day compared with the average findings of the 26 men who were taking part in the experiments which included these cases of heat prostration. The absolute magnitude of the pulse rate does not appear to have much significance but the failure to show an improvement from day to day must be considered as a danger sign.

The men who suffered heat exhaustion and prostration were kept in the high temperature suite. Treatment consisted solely of rest. This proved sufficient to enable all except one man to resume the work schedule within 24 to 30 hours. It is interesting to note that the men who suffered heat prostration or exhaustion did not exhibit excessive thirst at any time.

The Crampton scores for the four men who later suffered heat exhaustion were 5, 5, 25 and 30. Faintings before completion of observations were arbitrarily assigned a value of 5. Two men of the 22 who did not have heat exhaustion had scores of 5 and 30 and were classified by the observers as poor and two had scores of 30 and 25 and were classified as fair.

Evening Crampton scores of 30 or less may be classified as in the danger zone. Individual variations from day to day above this value are difficult to

TABLE 5

The per cent of subjects studied showing no improvement in pulse rate, rectal temperature and Crampton score after exposures to heat of different duration

DAYS IN HEAT COMPARED	NO. OF SUBJECTS STUDIED	PULSE	RECTAL TEMP.	CRAMPTON SCORE
		per cent	*per cent*	*per cent*
1st and 2nd day*.	43	21	68	40
1st and 3rd day*. .	17	12	24	12
1st and 4th day†. .	17	6	18	18
1st and 5th day*. .	6	0	0	0

* Basis of comparison p.m. values.

† Basis of comparison a.m. values.

TABLE 6

Comparison of the average values of work pulse rate, rectal temperature and Crampton scores on the evening of the first day in heat of 4 men who subsequently suffered heat exhaustion and the average values of 26 men exposed to identical conditions

	WORK PULSE RATE	RECTAL TEMP.	CRAMPTON SCORE
		°F.	°F.
Average of 4 men who suffered heat exhaustion	167	101.3	16
Average of 26 men who did not suffer heat exhaustion	166	101.3	43

interpret. Rank correlation coefficients of Crampton scores on repeat measurements under identical conditions after three preliminary trials were always poor (0.40). Morning Crampton scores did not appear to be a reliable index of condition.

We were unable to select those men who would tolerate heat well by any of the variables measured. No relationship was found in the cold between *a*, resting pulse rate; *b*, work pulse rate; *c*, Crampton score; *d*, basal rectal temperature; *e*, work rectal temperature (in the cold) and the ability to perform work in the heat either before or after acclimatization.

Discussion. The strain placed on the circulation by the excessive demands of the skin for an increased blood supply to serve the requirement of heat regulation may be very large. Adolph (6) has estimated that approximately 2.5 liters of blood per minute must be circulated through the skin to dissipate the heat production of walking in the desert. In the unacclimatized man this increased

circulation through the skin produces the early signs of peripheral circulatory collapse, i.e., a rapid work pulse rate (7), a decreased stroke volume and in some cases a decreased minute output (8), and a large increase in pulse rate and drop in blood pressure on assuming the upright position (9, 10). These facts are best explained by the inability of the vasomotor system to make the adjustments necessary to maintain complete filling of the great central veins. The rapid fall of the work pulse rate with continued exposure to heat of our subjects coupled with the marked improvement of the Crampton score indicates that the appropriate vasomotor adjustments are made in large part during the first four days in heat. The results of the cold pressor test, although not striking, suggest that an increased sensitivity of the vasomotor apparatus of the autonomic nervous system plays a rôle in the process of adaptation.

Blood volume changes may be of importance in this process. It is well known that loss of electrolytes and water will lead to a decreased blood volume (14). However, blood volume effects have been minimized by 1, an adequate salt intake, and 2, an adequate water intake. It is felt that the salt intake was adequate since doubling the salt intake did not influence the response or rate of acclimatization to heat (11).

Dill (1-B) has emphasized the rôle that an increased capacity to produce sweat plays in adaptation to high temperatures. The evidence presented here indicates that the 24-hour sweat production is not significantly changed and that the increased rate of sweating during work (see table 2) is a late and relatively minor adjustment. In fact, one-half of the total 26 per cent increase in the rate of sweating appears to serve no useful purpose since it occurred after all the adjustments in temperature regulation (as judged by the rectal temperature) had taken place.

Correlations between rectal temperature and pulse rate first reported by Haldane (12), between pulse rate and environmental temperature (13) and between the rate of sweating and pulse rate (6) will be dependent on the state of acclimatization and must be stated in terms of this variable.

SUMMARY

1. Seven thousand observations on pulse, blood pressure, rectal temperature and rate of sweating in work and rest are reported on 43 subjects (202 subject days) on a constant salt diet before and during exposure to dry heat for 2 to 8 days. Additional observations were made on 23 other subjects for 147 subject days. Observations of pulse and blood pressure before and after elevation on a tilt table were made morning and evening. Modified Crampton scores of cardiovascular fitness were calculated from these figures.

2. Marked deviations from control values in cool conditions were observed in work pulse rates, rectal temperatures and Crampton scores during the first days in heat.

3. Ten cases of heat exhaustion occurred; four of these were clear cut examples showing collapse with hypotension, tachycardia, vertigo and vomiting. Rest without removal from the hot environment sufficed to restore the ability to perform work in these men.

4. A rapid improvement in work pulse rate, rectal temperature and Crampton score took place and was complete in 4 to 5 days. No significant change took place in these variables from the fifth to eighth days.

5. The primary adjustment involved in acclimatization to heat is an improvement in cardiovascular efficiency. A decrease in the accumulation of heat, as measured by the rectal temperature during work, is probably secondary to cardiovascular improvement.

6. The average daily sweat loss is not affected by acclimatization. The rate of sweating during work tends to increase as acclimatization proceeds but a large part (one-half) of this change occurs after the more important adjustments, as indicated by the rectal temperature and pulse rate during work, have taken place.

7. The failure of the work pulse rate to show improvement over the value of the first day is a sign of impending heat exhaustion; similarly, poor cardiovascular postural adjustment in the evening is a danger sign.

8. None of the variables studied in the cold (control) are useful in the prediction of the ability to acclimatize in subsequent exposure in heat.

It is a pleasure to acknowledge the co-operation of Col. H. J. Keeley and Lt. Col. J. J. Shy and the men of the 710th M. P. Battalion. We are indebted to Miss Angie Mae Sturgeon, Head Technician, and Miss Evelyn Pearson, Dietitian, for many services in carrying out the routine of the experiments. We wish to thank Dr. Joseph Brozek for advice in the statistical analysis of the data.

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THE EFFECT OF EXPERIMENTAL HEPATITIS ON THE PLASMA PROTEINS OF THE IMMATURE RAT

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It has been shown that limitation of the diet during the added strain of pregnancy may reduce the protein content of the plasma and that in the presence of experimental hepatitis during pregnancy a marked lowering of the protein content of the plasma occurs. In these experiments it appeared that the drain of protein to the fetus was at the expense of the maternal plasma proteins and was reflected in the lowered level of protein in the plasma when the diet was restricted. Injury of the liver inhibited the formation of plasma albumin and further reduced the level of maternal plasma albumin. Experimental hepatitis and restriction of the diet of the nonpregnant rat are also associated with a lowered plasma protein. The extent of the lowering of the plasma proteins could not be so consistently correlated with the dietary restrictions of the nonpregnant animals as with those of the pregnant animals.

The added requirements for growth of the immature animal are somewhat similar to those of the pregnant animal. In a few preliminary experiments it was found that the administration of carbon tetrachloride to immature rats on restricted intake of food produced a lowering of the plasma proteins similar to that found after exposure to carbon tetrachloride of pregnant rats. The present series of experiments indicate that the protein content of the plasma of immature rats is lowered by the production of experimental hepatitis and that the lowering can be correlated with the amount of restriction of the diet. Attempts were also made to alter the levels of plasma protein in immature animals that had experimental hepatitis by altering the quality of the dietary protein. Unfortunately this phase of the work was interrupted by the war.

PROCEDURE. Individual litters of rats from twenty-one to twenty-six days of age were divided into two groups, one of which was to receive carbon tetrachloride and the other to serve as the control group. The experimental period differed among the various litters, but within each litter both the controls and the treated animals underwent the same regimen except for the carbon tetrachloride. The treated rats were exposed to carbon tetrachloride, 1 cc. vaporized in 10 liters of air, for a ten minute period each day. The average dietary intake of the controls was restricted to coincide with the average intake of the experimental animals each day. At the end of the experimental period the animals were weighed and anesthetized and blood was withdrawn from the heart. Pooled samples of each group were analyzed for total protein and for albumin and globulin by the method of Howe (1). The results of this experiment are given in table 1. All of the figures given are average figures obtained from the two to six rats in each experimental and control group. The food intake is recorded as the average daily intake for each rat.

There are obviously several objections to the procedure outlined in the preceding paragraph. Most of these objections were obviated in similar experiments repeated under paired feeding technic. Two animals of the same litter and the

TABLE 1

Effect of carbon tetrachloride on immature rats

EXPERIMENTAL PERIOD	BODY WEIGHT		LIVER WEIGHT		FOOD INTAKE		PLASMA TOTAL PROTEIN		PLASMA ALBUMIN		PLASMA GLOBULIN	
	Controls	CCl_4	Controls	CCl_4	Controls	CCl_4	Controls	CCl_4	Controls	CCl_4	Controls	CCl_4
days	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams per 100 cc.*	*grams per 100 cc.*	*grams per 100 cc.*	*grams per 100 cc.*	*grams per 100 cc.*	*grams per 100 cc.*
31	86.0	77.3	3.77	4.67	6.20	6.16	5.48	3.78	3.85	2.25	1.63	1.53
25	85.6	81.3	4.36	5.46	6.60	7.05	5.40	4.35	4.13	2.54	1.27	1.81
22	66.2	53.7	2.92	4.24	4.42	4.45	5.26	4.13	3.17	2.13	2.09	2.00
21	51.7	54.5	2.57	4.25	4.20	4.83	5.05	3.57	3.07	2.11	1.98	1.46
21	113.0	76.0	5.47	5.38	Ad lib	Ad lib	4.65	3.91	3.45	2.70	1.20	1.21
20	37.7	49.3	2.13	3.14	3.76	3.77	4.01	2.59				
20	57.3	60.0	3.45	4.24	4.05	5.20	4.90	3.84	3.09	1.87	1.81	1.47
18			2.54	5.41	5.40	5.32	5.22	3.60	3.68	2.26	1.54	1.34
18			2.34	3.49	4.25	4.84	4.61	3.88	2.67	2.22	1.94	1.66
18	44.4	45.4	2.25	3.98	4.75	4.70	5.13	4.15	3.64	2.74	1.49	1.41
17	46.5	46.0	2.01	3.59	4.20	4.71	4.84	3.63				
13	59.0	48.5	2.89	3.68	5.00	5.35	4.09	2.35				
12	39.4	32.8	1.81	2.68	3.74	2.92	4.16	3.88				

TABLE 2

Effect of carbon tetrachloride on immature rats: paired feeding technic

EXPERIMENTAL PERIOD	BODY WEIGHT		LIVER WEIGHT		DAILY NITROGEN INTAKE		PLASMA TOTAL PROTEIN	
	Control	CCl_4	Control	CCl_4	Control	CCl_4	Control	CCl_4
days	*grams*	*grams*	*grams*	*grams*	*mgm.*	*mgm.*	*grams per 100 cc.*	*grams per 100 cc.*
19	60	34	2.47	4.03	228	228	4.48	3.41
18	35	47*	1.72	3.40	212	212	5.62	3.19
19	50	46	2.17	3.74	188	188	3.45	2.90
19	47	34	2.25	3.81	183	183	5.40	2.94
18	61	56	2.84	3.76	173	173	3.62	2.88
14	34	48*	1.91	2.65	147	147	4.14	3.44
14	38	53*	2.79	3.26	146	146	4.33	3.62
18	36	54*	1.58	3.52	145	145	3.86	2.66
14	36	45*	1.58	3.28	118	118	4.13	3.60
10	37	27	2.14	1.76	89	89	4.88	2.10

* Ascites present.

same weight were kept in individual cages and allowed free access to water. The food was weighed individually and the dietary intake of each animal was determined by that of its pair mate. The results of this experiment are given in table 2. The nitrogen intake of each animal as given is the daily average for the

period of the experiment. During the first two or three days of the experiment the treated animals ate sparingly. The control animals were likewise restricted. As the experiment progressed, the intake of food was increased.

RESULTS. The livers of the animals receiving carbon tetrachloride were all definitely fatty in appearance in contrast to the normal red color of the livers of the control animals. The livers of the treated animals were also larger than those of the control animals. Hyperbilirubinemia was present in the treated animals and all of them appeared slightly icteric when carefully compared with the control animals. The body weight of the treated animals was less than that of the controls, except for those treated animals in which ascites developed. This may indicate that the utilization of food was impaired by the administration of carbon tetrachloride, as in many cases the intake of food of the treated animals exceeded that of their controls.

The rats which received carbon tetrachloride were unable to attain the levels of plasma protein maintained by the control animals. In comparable experiments the level of plasma protein was lowest in those treated animals receiving the greatest dietary restriction. It seems probable that the levels of plasma protein in the treated animals are the resultant of three components, the dietary intake, the protein requirements of the body and the injury to the liver. The changes produced in the globulin of the plasma were not great. In all cases the reduction of the level of plasma protein was at the expense of the albumin fraction.

Two other series of paired feeding experiments gave results very similar to those given in table 2. In these experiments an attempt was made to determine the effects of altering the quality of the protein in the diet by feeding a third rat for each pair a similar amount of diet except for the replacement of part of the diet with a different protein. In one series the replacement of 20 per cent of the protein with erythrocytes of dogs, a protein which is relatively poor for regeneration of plasma proteins after plasmapheresis (2), did not further lower the plasma protein content of the treated animals. In the other series the replacement of 30 per cent of the protein of the restricted diet with plasma protein did not appreciably increase the plasma protein of the treated animals. These experiments should be repeated with larger amounts of replacement of the diet with proteins thought to be especially favorable for plasma protein regeneration. The failure of these experiments to demonstrate any difference in effect of feeding proteins known to be good or poor for regeneration of plasma protein, however, indicates that the failure of production of plasma protein is due primarily to damage of the liver. The control experiments demonstrate that the quantity and quality of the proteins present in the diet were adequate for maintenance of the levels of plasma protein in the untreated animals.

SUMMARY

Experimental hepatitis produced by exposure of immature rats to carbon tetrachloride and restriction of diet reduces the level of protein of the plasma. The lowered level of plasma protein is almost entirely at the expense of the plasma albumin. There was a voluntary restriction of food intake by the treated animals

but much greater restriction of the diet of untreated animals did not produce comparable lowering of the plasma proteins. In the treated animals the level of protein in the plasma was roughly proportional to the dietary intake. The level of protein in the plasma, however, probably reflects the functional capacity of the liver more than the dietary intake. Substitution of protein, rated as good or poor for the production of plasma proteins in other experiments, for part of the dietary protein did not alter the levels of plasma protein in the treated animals.

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THE EFFECT OF EXPERIMENTAL HEPATITIS ON THE PLASMA PROTEINS OF THE PREGNANT RAT

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Factors influencing the regeneration of plasma protein have been successfully studied following plasmapheresis (1). While this method is well suited to larger experimental animals, unfortunately various difficulties make it less practicable for use in studies of the rat. In this regard, the idea presented itself that perhaps the physiologic stress of pregnancy might conceivably be considered as an "internal plasmapheresis." While it is true that "plasmapheresis" must here be construed in a modified sense of the term, since the characteristic replacement of whole blood by cells suspended in saline solution is completely lacking, yet it is appropriate to point out that the end result is of a similar nature. In both procedures there is a strain on the stores of body protein. Moreover, the fact that the strain of pregnancy is necessarily more prolonged than that of true plasmapheresis endows its use with special value. If now it be considered that there exists a dynamic equilibrium between the tissue stores of protein and the proteins of the blood stream, it becomes of interest to speculate concerning the effect of pregnancy on the level of the plasma proteins and, more particularly, whether or not the experimental hepatitis produced by administration of carbon tetrachloride during pregnancy might be reflected in the postpartum level of the plasma proteins.

PROCEDURE. *The effect of carbon tetrachloride during the gestation period.* Feeding experiments have been conducted on paired virgin rats obtained from an inbred stock. The animals were fed a commercial rat food which is adequate for rats. This food served as the basic diet throughout those experiments. After similar preliminary feeding periods designed to equalize the stores of tissue protein, the two animals were inseminated by males of the same stock. The presence of blood and mucus in the vaginal smear within a few days after insemination was indicative of the successful implantation of the ovum. One of the two pregnant animals was then give fifteen minute periods of inhalation of carbon tetrachloride daily during the last two weeks of pregnancy. The food intake of the animal so treated became the limiting factor for the intake permitted to its pair mate.

At the end of the gestation period, the newborn rats were weighed, and this weight subtracted from the maternal pregnant weight indicated the body weight of the adult animal alone. The maternal blood was removed by heart puncture, and the maternal livers were removed and weighed. It is obvious that there is a possibility of several variables in such an experiment. It is felt that these, however, have been controlled in great measure by the following precautions: the choice of virgin animals of similar weight from an inbred stock, the similar pre-

liminary feeding periods, the equal gestation periods, the equalized intakes of food, the production of young approximating the same total weight, and the removal of the maternal blood by the same procedure. Whenever any of these standards were not obtainable within reasonable limits, the experiment was discarded or repeated.

The effect of pregnancy alone. The same precautions were observed as previously. Animals of similar weight from an inbred stock were paired, and one

TABLE 1

The effect of carbon tetrachloride during the gestation period

ANIMAL	POSTPARTUM WEIGHT OF ANIMAL	LIVER WEIGHT	NUMBER OF YOUNG	WEIGHT OF YOUNG	TOTAL INTAKE OF FOOD	TOTAL PROTEIN	ALBUMIN	GLOBULIN	HEMATOCRIT
	grams	*grams*		*grams*	*grams*	*grams per 100 cc.*	*grams per 100 cc.*	*grams per 100 cc.*	*per cent*
1	202	7.97	8	32	243	5.35	3.42	1.93	34.7
1 (CCl_4)	193	10.80	6	21	243	4.89	2.67	2.22	34.2
2	168	7.02	7	34	165	5.81	4.05	1.76	32.4
2 (CCl_4)	153	9.00	8	40	153	4.87	3.10	1.77	39.2
3	171	6.87	5	35	137	4.74			35.0
3 (CCl_4)	171	7.15	8	41	137	4.35			35.0
4	171	6.87	5	35	137	4.74	3.46	1.28	35.8
4 (CCl_4)	172	7.72	8	42	151	3.73	1.86	1.77	40.7
5	172	6.33	6	21	137	4.92	3.27	1.65	35.5
5 (CCl_4)	193	10.80	6	21	243	4.89	2.67	2.22	34.2
6	169	7.30	9	43	139	4.80	3.09	1.71	34.9
6 (CCl_4)	162	6.99	8	32	128	3.88	1.76	2.12	38.9
7	308	11.85	9	24	375	5.59	3.70	1.89	50.5
7 (CCl_4)	302	14.26	12	46	468	5.35	2.25	3.10	35.1
8	220	10.88	5	27	Ad lib	6.58	3.45	3.13	34.1
8 (CCl_4)	192	10.83	6	18	Ad lib	5.00	3.20	1.80	37.9
9	233	11.14	9	33	218	4.86	2.90	1.96	34.8
9 (CCl_4)	262	13.59	9	33	419	4.07	2.51	1.56	36.1

was chosen for insemination. The food intakes were equilibrated during the experimental period, and following the end of gestation, the body weights, liver weights and blood were obtained in the manner described in the preceding section.

RESULTS. *The effect of carbon tetrachloride during the gestation period.* The usual result of treatment with carbon tetrachloride during pregnancy was to cause lower total protein levels than were present when no carbon tetrachloride had been administered. Hypertrophy of the liver indicated that hepatic damage had occurred in the animals treated with carbon tetrachloride. The decrease of

total plasma protein was due to a specific lowering of albumin and not to any decrease of globulin. In fact, the serum globulin of the animals that had cirrhosis was frequently increased, so that the albumin globulin ratio either approached unity or became less than unity. No consistent effect on the hematocrit was demonstrable. These results are presented in table 1. Perusal of this table indicates that the results can be classed roughly in two groups: 1, those in which approximately the same intake of food was not capable of maintaining the total plasma protein as high when carbon tetrachloride had been administered as when it had not been administered, and 2, those in which, in order to attain nearly the same total plasma protein concentration as its pair mate, the animal that received carbon tetrachloride required much greater quantities of food than its pair mate. Even then, the increase was usually limited to the globulin fraction, and the albumin was not increased by these measures.

TABLE 2

The effect of pregnancy

ANIMAL	POSTPARTUM WEIGHT OF ANIMAL	LIVER WEIGHT	NUMBER OF YOUNG	WEIGHT OF YOUNG	TOTAL INTAKE OF FOOD	TOTAL PROTEIN	ALBUMIN	GLOBULIN	HEMATOCRIT
	grams	*grams*		*grams*	*grams*	*grams per 100 cc.*	*grams per 100 cc.*	*grams per 100 cc.*	*per cent*
1 P	171	6.87	5	35	137	4.74	3.46	1.28	35.8
1 NP	178	6.87	0		142	6.23	3.46	2.77	41.6
2 P	169	7.30	9	43	139	4.80	3.09	1.71	34.9
2 NP	154	5.03	0		128	5.26	3.49	1.87	41.7
3 P	172	6.33	6	21	137	4.92	3.27	1.65	35.5
3 NP	175	5.75	0		137	5.52	3.39	2.13	44.7
4 P	233	11.14	9	33	218	4.86	2.90	1.96	34.8
4 NP	222	9.44	0		218	6.47	3.57	2.88	37.0
5 P	202	7.97	8	32	243	5.35	3.42	1.93	34.7
5 NP	200	7.93	0		243	6.27	3.43	2.84	39.5

P = pregnancy, NP = no pregnancy.

The gross appearance of the newborn offspring of the animals treated with carbon tetrachloride was no different from that of the newborn not exposed to this toxin. Histologically, the livers of the young whose mothers had received carbon tetrachloride showed only slight differences, which were not consistent.

The effect of pregnancy alone. The effect of pregnancy alone on the concentration of the plasma proteins was to produce levels lower than were present in nonpregnant animals. In other words, as might be expected, pregnancy is a drain on the maternal store of protein, which is reflected in the levels of the total protein concentration, when the food intake has been limited. Contrary to the findings for the rats treated with carbon tetrachloride, this decrease was not of albumin but of globulin (table 2). The effect of pregnancy was also to decrease the hematrocrit but there was no consistent effect on hypertrophy of the liver.

SUMMARY

Carbon tetrachloride administered to rats during pregnancy causes a lowering of the maternal plasma total protein and the albumin existing immediately postpartum, as compared with pair mates not receiving carbon tetrachloride. The globulin fraction is, on the contrary, frequently increased. Some hypertrophic changes occur in the liver as a result of administration of carbon tetrachloride.

The effect of pregnancy alone, under the conditions of these experiments, is to cause a lowering of the globulin fraction, which is reflected in the total protein level. The concentration of albumin is not markedly affected. There is no demonstrable hypertrophy of the liver.

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MOTOR CONTROL OF THE THORACIC DUCT

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Bert and Laffont (1882) showed that electrical stimulation of the mesenteric nerves caused a contraction of the lacteals in a digesting animal, while stimulation of the splanchnic nerves produced a dilatation. Furthermore, stimulation of the vagus resulted in a brief lympathic dilatation followed by a constriction. Mechanical stimulation of the thoracic duct or of the lacteals elicited a wave of contraction which progressed slowly and which was followed by a wave of dilatation.

Camus and Gley (1894), working on dogs with a perfused thoracic duct, reported that stimulation of the left splanchnic caused a dilatation of the cisterna and a corresponding decrease of its internal pressure. They (1895a) also showed that stimulation of the left thoracic sympathetic chain below the stellate ganglion produced a dilatation of the duct. They concluded that the sympathetic elements in the left thoracic chain and splanchnic nerve include dilator fibers for the duct and the cisterna, respectively. In some of their observations stimulation of these nerves caused a constriction. This discrepancy was explained by the assumption that there are also sympathetic constrictors. Stimulation of the vagus by these observers (1895a) led to a decrease of flow through the thoracic duct in only one instance, which they considered atypical. Pilocarpine, they (1895b) found, caused a constriction of the thoracic duct while atropine had a dilator effect.

Beznák (1937) noted that acetylcholine increases the flow of lymph through the duct. Since the microscopic observation of the intestinal villi revealed a concomitant dilatation of arterioles and capillaries he concluded that the increased thoracic flow was due to an enhanced production of lymph.

The survey of the literature shows that the knowledge of the motor control of the thoracic duct is incomplete. The present observations were made with the purpose of determining the influence of the vagi and of acetylcholine and adrenaline on the motility of the duct and cisterna.

METHOD. Cats were used, anesthetized with dial (Ciba, 0.7 cc. per kgm., intraperitoneally). The animals were kept fasting for 24 hours, and then, two hours before the experiment, were given 60 cc. of olive oil through a gastric tube. In addition, after they were anesthetized, they received an intravenous injection of 10 cc. of dextrose (50 per cent), in order to distend the thoracic duct with lymph. A tracheal cannula was inserted and artificial respiration was administered.

A small segment of the innominate vein, at the region of entrance of the

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thoracic duct, was isolated by ligatures of that vein and all of its branches. A cannula was then inserted into the cisterna, which was approached by a midline abdominal incision and by section of the diaphragm. A mass ligature eliminated all the connections of the cisterna other than that with the duct. The innominate vein was then cannulated and perfusion of the duct was started.

The perfusing fluid was Tyrode solution with 6 per cent arabic gum and at 37°. The pressure (from 30 to 50 cm. of the solution) was regulated by a Mariotte flask and was adjusted to obtain 6 to 9 drops per minute. The drops fell on a Marey tambour connected to another tambour recording on the kymograph.

The thorax was opened widely in order to eliminate changes in the diameter of the cisterna or duct due to respiratory activity. In these conditions a contraction of the lymphatic channels will result first in an increase of the flowing drops due to a decrease of capacity, and then in a decrease of flow, due to increased resistance. Conversely, a dilatation will appear as a decrease followed by an increase of the flow.

Results. In a series of observations acetylcholine or adrenaline was added to the perfusing fluid. The solutions were slowly injected into rubber tubing near the cannula inserted in the cisterna. The addition of acetylcholine (10^{-5} to 10^{-7}) resulted in a decrease of flow through the duct (fig. 1). The latency of the effect was variable but its duration was usually prolonged. It may be inferred that acetylcholine has a constrictor action on the main lymphatic trunk.

Similar injections of adrenaline caused an increase of flow, thus indicating that the hormone has a relaxing action (fig. 2). This relaxing action was also observed when adrenaline was administered in the course of the constrictor response to acetylcholine.

The responses both to acetylcholine and to adrenaline decreased in the course of an experiment; after a few hours they were minimal or negligible. They also decreased considerably if the perfusing fluid became cooler.

In another series of observations the effects of intravenous injections of acetylcholine or adrenaline were recorded. Acetylcholine (0.1 γ) caused a decrease of flow, much as it did when added to the perfusing fluid. This decrease was sometimes preceded by a brief increase which may be attributed to a reduction of capacity (p. 602). Injections of adrenaline (20 γ) resulted in an increase of flow preceded sometimes by a brief decrease. Occasionally adrenaline elicited exclusively a decrease of flow, not followed by an increase. This effect may be explained by assuming an inhibitory action affecting exclusively the cisterna—not the duct.

In a final series of observations the vagi were dissected in the neck and were cut centrally. Electrodes were applied to the peripheral parts. Stimulation with faradic shocks from a Harvard coil with 1.5 volts in the primary circuit, was followed by a decrease of flow. It may be inferred that the vagal nerve impulses, like acetylcholine, exert a constrictor influence.

Comment. It is usually assumed that the main factor which regulates the flow of lymph through the thoracic duct is the difference between the positive abdominal and the negative intra-thoracic pressures. There is, however, ana-

tomical evidence that the walls of the cisterna and duct contain smooth muscle elements. In addition Quénu and Darier (1882) described a nervous plexus of fine nonmyelinated fibers in the walls of the thoracic duct of the dog. These anatomical data suggest contractile functions under a nervous control. The present observations confirm this expectation. Unlike blood vessels, however, which as a rule constrict in response to sympathetic impulses, the thoracic duct and cisterna relax under this influence and contract in response to parasympathetic stimuli.

Three factors determine the flow of lymph through the thoracic duct: a, the "vis a tergo," which depends on the continuous production of lymph; b, the

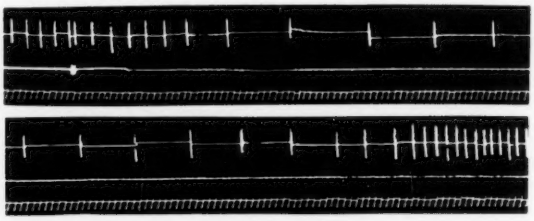

Fig. 1. Decrease of flow through the perfused thoracic duct caused by acetylcholine. Upper record: drops of perfusing fluid. Middle signal: injection of acetylcholine (1γ) into the fluid. Lower signal: 5 sec. intervals. The lower strip follows the upper one immediately.

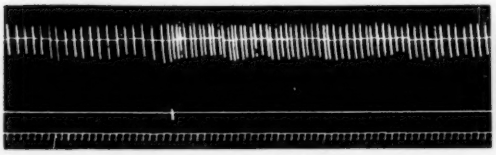

Fig. 2

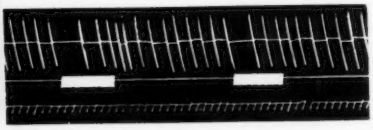

Fig. 3

Fig. 2. Increase of flow caused by adrenaline. As in figure 1, but at middle signal adrenaline (10γ) added to the perfusing fluid.

Fig. 3. Decrease of flow caused by vagal stimulation. As in figure 1, but at the middle signals, first the left and then the right vagi were stimulated peripherally with a coil distance of 12 cm.

pressure gradient between the abdominal and thoracic cavities; and c, the motility of the cisterna and thoracic duct controlled by the autonomic nervous system.

SUMMARY

The cisterna chyli and the venous ending of the thoracic duct were cannulated in cats. The flow through these channels was measured by recording the number of drops per unit time with a constant head of pressure of a perfusing fluid.

When acetylcholine was added to the perfusing fluid, the flow decreased, indicating a constrictor effect (fig. 1). Conversely, perfusion with adrenaline resulted in an increased flow, which showed a dilator action (fig. 2). Intravenous

injection of these substances produced effects similar to those obtained when they were added to the perfusing fluid. Stimulation of the vagus nerve, like acetylcholine, had a constrictor action (fig. 3).

In confirmation of early studies it is inferred that the lymph channels are contractile organs under nervous control. This contractility is one of the factors which governs the flow of lymph.

I wish to express my appreciation to Dr. Walter B. Cannon for his kindly hospitality during my stay at his department and to Dr. Arturo Rosenblueth for his help in the preparation of this paper for publication.

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ADRENAL CORTEX AND BLOOD PRESSURE RESPONSE TO CARBON ARC IRRADIATION[1, 2]

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In normal dogs large doses of carbon arc radiation produce a fall in blood pressure (1). This reduction cannot be explained by changes in blood volume (2) or in cardiac output (3), but is believed to be due to a decrease in peripheral resistance brought about by arteriolar dilatation (4). Many observers believe that histamine is set free in the irradiated tissues, absorbed and circulated, producing an extensive arteriolar dilatation which lowers the blood pressure (5). A correlation between an increased content of H-substance in the blood and a reduction in blood pressure following irradiation has been reported (6).

In the absence of the adrenal glands there is a marked impairment of the ability to destroy or inactivate histamine, rendering an adrenalectomized animal many times more susceptible to the effects of histamine than a normal one (7, 8). This resistance to histamine can be restored to normal by the administration of adequate amounts of adrenal cortical substances (9). If the circulatory changes following carbon arc irradiation are due to the production or release of histamine, then such changes should be magnified in an animal possessing no adrenal cortex. To test this hypothesis, dogs were irradiated before and after bilateral adrenalectomy.

PROCEDURE. Adult dogs, ranging in weight from 7 to 14 kilos, were trained to lie quietly on a table for varying periods of time until no excitement was exhibited during blood pressure determinations, withdrawal of blood samples, or operation of the carbon arc lamp. When the period of training was completed, blood pressure, hematocrit, blood sugar, and plasma potassium values were determined until control levels were established.

The auscultatory method of determining blood pressure was employed, the values being recorded from the hind limb. The source of radiation was an Eveready professional model carbon arc lamp, emitting 0.820 gram calorie per square centimeter per minute with a distribution of 5–6 per cent ultraviolet, 29–31 per cent luminous, and 63–66 per cent infra red. The distance of the lamp from the animals and the time of exposure were so adjusted that all experiments were performed with a dosage of 40 gram calories per square centimeter. Ex-

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[2] Aided by a grant from the Committee on Scientific Research of the American Medical Association to Prof. Henry Laurens. The National Carbon Company loaned the lamps and furnished the carbons. The Ciba Pharmaceutical Products, Inc. and Roche-Organon, Inc. were generous in their gifts of desoxycorticosterone acetate. Roche-Organon, Inc. and the Upjohn Company gave ample supplies of adrenal cortical extract.

posures were made upon the abdominal region of the dog's body after careful shaving and cleansing of the skin.

The same general procedure was followed in all experiments. The dogs were brought into the laboratory early in the morning, shaved, and placed upon the table. The blood pressure was determined at fifteen minute intervals until two similar consecutive values were obtained. A blood sample was withdrawn, a final blood pressure reading made, and the animal then irradiated. After the irradiation blood pressure readings and blood samples were taken at one or two hour intervals throughout the day. After two or three control experiments, the dogs were doubly adrenalectomized, the operation being performed in two stages under aseptic conditions. The animals were maintained, after adrenalectomy, on desoxycorticosterone acetate (Ciba, Roche-Organon) supplemented at times with adrenal cortical extract (Upjohn, Roche-Organon). Frequent determinations of the blood sugar and potassium as well as blood pressure were the criteria of adequate replacement therapy. After full recovery from the second operation had occurred, the irradiation experiments were repeated exactly as before.

A total of twenty-nine control experiments were performed on eleven dogs. Thirty-eight experiments were repeated on six of these dogs after bilateral adrenalectomy. Twenty-nine experiments were performed on six control animals after the administration of "priming" doses of desoxycorticosterone acetate (D. C. A.) or adrenal cortical extract (A. C. E.). Two dogs which were used in the pretreatment experiments were made hypertensive by application of Goldblatt clamps and again exposed to the carbon arc lamp before and after the administration of D. C. A.

Results. Protocols of typical experiments are shown in table 1. In the control experiments (fig. 1A) the blood pressure began to decline during the first hour following exposure of the animals to the carbon arc. The decline was progressive for six hours following irradiation with recovery gradually taking place after this time. There was a greater proportionate reduction and a more delayed return to normal in the diastolic than in the systolic pressure.

After removal of one adrenal gland the blood pressure response was not appreciably altered from that seen in control experiments (fig. 1C). Following bilateral adrenalectomy, however, a profound increase in the effect of irradiation was produced (fig. 1D). This mainly affected the diastolic pressure; the systolic readings when averaged were almost identical with the values obtained before removal of the adrenal glands. In the instance of the diastolic pressure the average maximal reduction of 23 per cent, occurring six hours after irradiation in the control animals, gave way to an average maximal reduction of 63 per cent occurring five hours after the exposure. In individual experiments the actual reduction of diastolic pressure was often greater than average, and in one instance the animal died thirty-two hours after the irradiation. In this case there was a progressive decrease in diastolic pressure to shock level, an absence of recovery as usually seen, and a failure of the systolic pressure to be maintained. The animal died exhibiting typical symptoms of shock.

It was observed during these experiments that in some instances the adrenal-

TABLE 1

TIME	BLOOD PRESSURE	PULSE RATE PER MIN.	HEMATOCRIT	BLOOD SUGAR	PLASMA POTASSIUM
	Experiment 1. 8/19. Control				
	mm. Hg		*per cent*	*mgm. per cent*	*mgm. per cent*
9:00 a.m.	140/84	68	34	85.8	20.6
	Irradiation fifty minutes with carbon arc at one meter				
11:00 a.m.	126/72	76	34	88.2	21.2
12:00 m.	118/68	78	35	80.4	20.8
1:30 p.m.	110/68	76	33	84.8	20.8
2:30 p.m.	108/60	76	32	80.7	19.8
3:30 p.m.	108/62	78	32	80.0	21.6
5:30 p.m.	122/68	72	34	86.7	21.0
24 hrs.	142/80	70	35	83.4	22.0
	Experiment 4. 9/12. After pretreatment				
9:00 a.m.	146/78	66	34	88.6	21.0
	Desoxycorticosterone acetate, 20 mgm., administered intramuscularly. Irradiation fifty minutes with carbon arc at one meter				
11:00 a.m.	148/82	70	35	85.2	19.8
12:00 m.	148/76	74	34	86.0	22.4
1:00 p.m.	152/84	78	34	86.4	22.1
3:00 p.m.	150/80	76	36	88.0	22.0
5:00 p.m.	150/84	70	35	88.0	21.7
9:00 p.m.	148/80	70	34	86.9	19.4
24 hrs.	148/82	70	34	86.2	20.9
	Experiment 8. 10/22. After unilateral adrenalectomy (10/7)				
9:00 a.m.	142/76	62	33	81.9	21.7
	Irradiation fifty minutes with carbon arc at one meter				
11:00 a.m.	136/62	68	34	80.6	21.4
12:00 m.	128/56	74	34	84.5	20.6
1:00 p.m.	116/50	76	33	86.3	22.0
2:00 p.m.	112/50	76	33	84.0	19.4
3:00 p.m.	112/50	74	32	80.1	19.6
4:00 p.m.	110/52	74	30	80.9	20.4
5:00 p.m.	112/56	70	30	79.6	20.4
8:30 p.m.	118/64	70	32	82.8	22.8
24 hrs.	148/72	66	35	86.4	22.1
	Experiment 12. 12/27. After double adrenalectomy (12/1). D. C. A. discontinued 48 hours				
9:00 a.m.	150/82	68	41	78.6	21.3
	Irradiation fifty minutes with carbon arc at one meter				
11:00 a.m.	142/70	72	41	78.2	19.3
12:00 m.	130/62	84	38	82.9	21.8
1:00 p.m.	122/42	86	37	84.4	20.7
2:00 p.m.	116/28	88	36	70.7	22.4
3:00 p.m.	110/26	96	35	79.3	21.6
4:00 p.m.	106/18	96	35	76.8	19.5
7:00 p.m.	106/24	84	36	78.5	19.8
9:00 p.m.	118/52	76	38	79.6	21.2
24 hrs.	144/86	70	38	83.2	20.9

ectomized animals failed to show as great a reduction in blood pressure as the controls. This seemed to be correlated with the length of time elapsing between the administration of the maintenance dose of D. C. A. and the beginning of the experiment. Accordingly, it was found that when the animals received D. C. A. up to and including the day of the experiment no great reduction in blood pressure

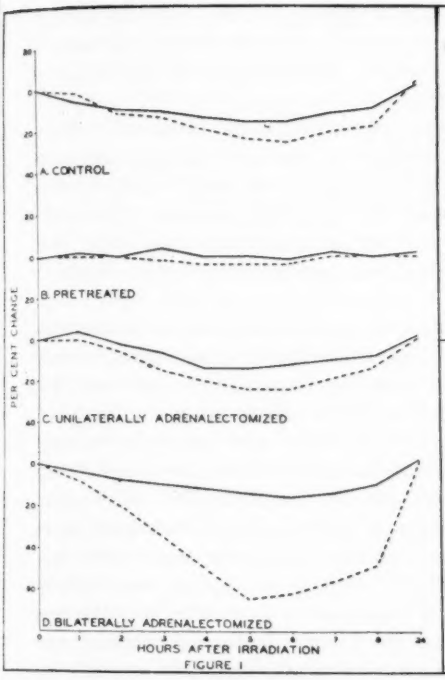

FIGURE 1

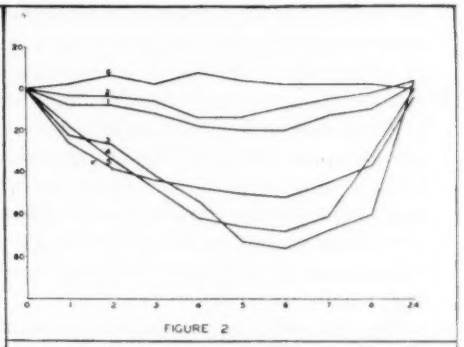

FIGURE 2

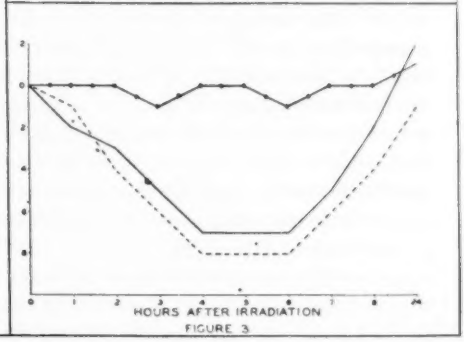

FIGURE 3

Fig. 1. Curves showing per cent change in blood pressure following carbon arc irradiation. Solid lines indicate systolic, dotted lines diastolic blood pressure.

Fig. 2. Diastolic blood pressure following carbon arc irradiation. *1*, control; *2*, adrenalectomized, D. C. A. not discontinued; *3*, adrenalectomized, D. C. A. discontinued 24 hrs.; *4*, adrenalectomized, D. C. A. discontinued 48 hrs.; *5*, adrenalectomized, D. C. A. discontinued 5 days; *6*, adrenalectomized, D. C. A. discontinued 5 days, with 10 mgm. received on day of experiment.

Fig. 3. Hematocrit changes following carbon arc irradiation. —— control, ---- bilaterally adrenalectomized, —○—○— pretreated animals.

occurred. The alterations in blood pressure response to irradiation after withholding the maintenance dose of D. C. A. for varying periods of time before the experiment are represented in figure 2. Symptoms of adrenal insufficiency did not appear in most instances until the drug had been discontinued for ten days to two weeks. These observations prompted the experiments on normal animals pre-treated with large doses of D. C. A. or A. C. E. before irradiation. The same

plan as outlined for the control experiments was followed. The irradiations were then repeated after the administration of D. C. A. or A. C. E. (fig. 1B). Such a procedure prevented changes in both systolic and diastolic pressures by the action of the carbon arc. No attempt was made to determine the least amount or optimal dosage of the materials used to produce this effect. It was found that A. C. E. was as efficacious as D. C. A. provided it was administered on the day of the experiment; intravenous administration of the extract twelve hours before experimentation gave no protection to the animal. Intramuscular injection of D. C. A. from one to twelve hours before exposure to the carbon arc was effective in preventing the fall in blood pressure.

In the experience of other workers, carbon arc irradiation has its greatest effect on blood pressures which are initially elevated above the upper limits of normal; for this reason two of the animals used in the experiments just described were made hypertensive. After the establishment of high blood pressures, these animals were irradiated before and after the administration of D. C. A. The responses were essentially the same as those seen at the lower levels of blood pressure.

Figure 3 represents a composite graph of hematocrit changes in all experiments. The dosage of carbon arc irradiation used produced in the normal animal a reduction in red cell concentration, commencing immediately following the exposure, and reaching, after four hours, a maximum which was maintained for the next two hours. Recovery then began to take place and twenty-four hours later there was a normal or slightly higher than normal concentration of red blood corpuscles. After removal of both adrenal glands, the hematocrit determinations followed the same general course from hour to hour as in the control experiments, with no significant intensification of the decrease excepting some delay in recovery to the original values. A higher than initial reading was seldom seen in the adrenalectomized animals twenty-four hours after irradiation, whereas this was usually observed in the normal dog. In normal animals pretreated with adrenal cortical substances there were no significant changes in hematocrit reading following irradiation. This was also true in adrenalectomized animals which were irradiated without a discontinuation of the D. C. A.

Blood sugar and plasma potassium values showed no alterations that were not within the limits of normal physiological fluctuations. The blood sugar in many experiments tended to decrease during the period of maximal blood dilution as indicated by the hematocrit determinations. Fluctuations in plasma potassium followed no general trend and were not regarded as significant.

Discussion. Direct evidence and studies which eliminate other factors have shown that the cause of the fall in blood pressure following carbon arc irradiation is a vasodilatation of the peripheral vessels. This might be produced by reflex nervous mechanisms or by the action of a vasodilator substance. In the light of recent evidence, the protective action of both D. C. A. and A. C. E. in the normal as well as the adrenalectomized animals becomes significant in an analysis of this phenomenon.

From the results obtained it is evident that absence of the adrenal glands

markedly increases the effect of carbon arc radiation on blood pressure. It should be emphasized that the reduced pressure was not accompanied by any change in blood sugar or plasma potassium; the irradiation, therefore, did not produce a typical adrenal insufficiency crisis. These results are in agreement with the concept of Swingle and his co-workers (10, 11) who believe that the secretion of the adrenal cortex is in some way necessary for maintaining the normal ability of the periphery to cope with a vascular strain. Swingle et al. found that large doses of D. C. A. were effective in preventing the shock resulting in adrenalectomized dogs from muscle trauma, intraperitoneal injection of an isotonic solution of glucose, or the injection of large amounts of epinephrine. In contrast, no evidence of a protective action was found against the shock following intestinal stripping in these animals or the circulatory failure following one stage bilateral adrenalectomy. Adrenal cortical extract was effective in preventing shock following any of the procedures. Similar results were obtained by Selye et al. (12) with intact rats, and by Weil and associates (13) with normal rabbits. Swingle and co-workers subsequently showed that the circulation of dogs could be maintained with D. C. A., as well as with A. C. E., following one stage bilateral adrenalectomy provided that the operation was preceded by local blocking of the nerves in the operative field with procaine, by the use of spinal anesthesia, or by section of the spinal cord at the first thoracic vertebra. Spinal section also prevented the circulatory failure following intestinal stripping, and the blood pressure was well maintained by D. C. A. The results seem to indicate the participation of a nervous factor in the circulatory collapse resulting from the procedures. The difference in action of D. C. A. and A. C. E. under these circumstances has been ascribed to the inability of D. C. A. to maintain normal carbohydrate metabolism, resulting in a failure of the peripheral vasculature consequent to the intense reflex stimulation. The frequent occurrence of hypoglycemia under these conditions and the finding that in all types of shock-inducing procedures employed the protective action of D. C. A. was diminished if the adrenalectomized animal had been maintained exclusively on D. C. A. for a long period of time have strengthened this point of view. In the present study, such an explanation for the prophylactic action of adrenal cortical compounds does not seem applicable. D. C. A. was just as effective as A. C. E. in maintaining the circulation in both adrenalectomized and normal dogs. In unprimed animals a hypoglycemia was not associated with the fall in blood pressure.

In view of the complete protection afforded our animals by D. C. A. we do not believe a nervous factor or a disturbance in carbohydrate metabolism is involved. Katz (14) found that the shock produced by massive venous occlusion of the leg in dogs was prevented by pretreatment with D. C. A. In these experiments the rate of fluid loss from the blood stream was diminished by D. C. A., but the ultimate total loss of fluid was not appreciably changed. The protective action of D. C. A. in this instance could not be solely explained by its effect on loss of fluid. In our experiments, D. C. A. and A. C. E. afforded protection against blood pressure changes which appeared in the presence of a blood dilution rather than a hemoconcentration.

It has been suggested (2) that a decrease in the hydrostatic pressure within the blood vessels consequent to a vasodilatation is responsible for the blood dilution. The passage of fluid into the blood stream is insufficient to increase the blood volume enough to restore the blood pressure. This may be associated with a simultaneous increase in the capillary permeability allowing the escape of some of the plasma protein, thereby diminishing to some extent the exchange of fluid. That the adrenal cortical substances did not maintain the blood pressure by allowing a more complete compensation is shown by the close correlation between the hematocrit and blood pressure values. Both begin to decrease in the first hour after irradiation. Both maintain their maximum reductions for about the same time, and the recovery to normal begins almost simultaneously. In the absence of any blood pressure changes there are no significant changes in the hematocrit. Adrenal cortical extract and D. C. A. apparently prevent the initiating factor for such changes, i.e., vasodilatation.

Much evidence has accumulated which indicates that carbon arc irradiation results in the production and release of a vasodilator substance from the irradiated tissues (5, 6). The most probable explanation of the effect of D. C. A. and A. C. E. in these experiments seems to be the protection which such substances are known to exert against the toxic manifestations of histamine. Certainly, whatever substance is produced, it has one property in common with histamine: its action is intensified in the absence of the adrenal cortex and is diminished in the presence of excessive amounts of adrenal cortical substances. It may well be that we are dealing with a complex series of events in which the release, or production, and action of histamine comprise but one important and necessary step in the achievement of the end result.

SUMMARY

1. Irradiation of normal dogs with a carbon arc lamp, using a dosage of 40 gram calories per square centimeter, produced a fall in blood pressure averaging 16 per cent in systolic and 23 per cent in diastolic. After removal of both adrenal glands this reduction averaged 16 per cent for systolic and 63 per cent for diastolic. The fall in pressure could be prevented in normal animals and diminished or prevented in adrenalectomized animals by the administration of D. C. A. or A. C. E.

2. The red blood cell concentration was reduced by an average of 7 per cent following irradiation of the normal dog. After adrenalectomy irradiation reduced the red blood cell concentration by an average of 8 per cent. This reduction, like that of the blood pressure, could be prevented by the administration of adrenal cortical substances.

3. No significant changes in blood sugar or plasma potassium concentration were encountered.

4. It is suggested that the evidence presented further substantiates the view that a humoral mechanism is responsible for the reduction in blood pressure following exposure to the carbon arc lamp.

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THE BIOASSAY OF HEPARIN PREPARATIONS

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In the preparation of heparin it is essential to have available an adequate assay method for the determination of the anticoagulant activity of the various fractions obtained. The cat method of assay described by Howell (1) and used by Scott and Charles (2) has been of great value, but is limited in its application because of the variability of the results obtained with individual cats. The various factors that may influence the results have been enumerated by Scott and Charles (2). We have employed the cat method for assaying heparin preparations of varying degrees of activity and found that evaluation of an unknown in terms of the standard was very difficult. This difficulty is due principally to the fact that greater than one hundred per cent increases in heparin concentration are necessary to differentiate between clotted and fluid samples of cat blood.

Because of these difficulties, methods have been described using beef (3) or horse (4) citrated or oxalated plasmas. In a study of citrated plasmas as clotting systems for the measurement of heparin activity, we have found that the best results can be obtained by the use of citrated sheep plasma. The purpose of this paper is to describe a sheep plasma method of relatively high sensitivity for comparing two or more heparin solutions, one of which can be taken as the standard. It has been suggested by Murray and Best (5) that the crystalline barium salt of heparin be assumed to contain 100 units per milligram and used as a standard. The homogeneity and therefore the constancy of anticoagulant activity of this material has been questioned (2, 6). It was subsequently suggested that the barium-free material be taken as a standard (7). Since this confusion exists relative to the choice of a standard we have decided to use the purified sodium salt of heparin put out by the Connaught Laboratories, in solution of 10 mgm. per cc. Several lots of this preparation checked against each other were found to have identical anticoagulant activity. The standard used for the experiments reported here was made from the two lots 86-1 and 87-1.

EXPERIMENTAL. Whole cat blood, recalcified citrated beef plasma, horse plasma and sheep plasma[1] were studied for their suitability as coagulation systems in the assay of heparin.

The cat method of assay. Two sodium salts were assayed by the cat method according to the procedure of Scott and Charles (2). In table 1 are given the results with five cats. Various dilutions of the heparin were made so that when 0.3 cc. was placed in the tube and 0.7 cc. of blood added the assignment of the even whole numbers 100 to 600 could be made. Completely fluid samples are

[1] We are indebted to Dr. David Klein of the Wilson Laboratories for supplying the sheep plasma.

designated Fl and completely clotted samples Cl. Samples which on inversion slowly ran down the sides of the tube, but were partially clotted, were designated Fl?, and those that were mostly clotted but also could be made to run down the sides with a little fluid present were designated Cl?. The long range of questionable coagulation with the cat blood is apparent from the table. In some instances to go from a fluid to a clot a six-fold dilution of heparin was necessary. The difficulty of evaluating an unknown in terms of the standard by the cat method of assay is therefore obvious. This prompted an investigation of plasma methods for the assay of heparin.

Comparison of horse, beef and sheep plasma clotting systems. For use as a clotting system, the citrated plasmas of horse, beef and sheep have the definite advantage that they can be collected in quantity and stored in the frozen state until ready for use. An ideal plasma for use in the assay of heparin should have the following characteristics: First, the normal clotting time on recalcification

TABLE 1

The assay of sodium salt of heparin by the cat method

UNITS PER MGM.	CAT 6			CAT 7			CAT 8			CAT 9			CAT 10		
	Standard	81A-9	82-9	Standard	81A-9	82-9	Standard	81A-9	82-9	Standard	81A-9	82-9	Standard	81A-9	82-9
100	Fl	Fl	Fl	Fl	Fl	Fl	Fl	Fl	Fl	Fl	Fl	Fl	Fl	Fl	Fl
200	Fl	Fl	Fl	Fl?	Fl?	Fl?	Fl	Fl	Fl	Fl	Fl	Fl	Fl?	Fl	Fl
300	Fl?	Fl?	Fl?	Fl?	Fl?	Fl?	Fl	Fl?	Fl?	Fl	Fl	Fl	Fl?	Fl	Fl
350	Cl?	Cl?	Cl?	Fl?	Fl?	Fl?	Fl?	Cl?	Cl?	Fl	Fl	Fl	Fl?	Fl?	Fl?
400	Cl?	Cl?	Cl?	Fl?	Fl?	Fl?	Cl?	Cl?	Cl?	Cl?	Fl?	Fl?	Fl?	Cl?	Cl?
500	Cl	Cl	Cl	Cl?	Cl?	Cl?	Cl	Cl	Cl	Cl	Cl?	Cl?	Cl?	Cl	Cl
600	Cl	Cl	Cl	Cl	Cl	Cl	Cl	Cl	Cl	Cl	Cl	Cl	Cl	Cl	Cl

should be short,—not greater than three minutes; second, individual samples from the same species should give nearly the same clotting time and, third, there should be a sharp differentiation between clotted and fluid samples at increasing concentrations of heparin. Since normal clotting time did not vary for a period of one year when plasmas were stored at −10°C., all samples were kept at this temperature.

Of 50 individual samples of horse plasma which were available to us, only two were found on recalcification to have clotting times of three minutes or less. The individual variation was very great and therefore the range of heparin concentration would be greatly different for each batch of plasma. Beef and sheep plasmas have desirable coagulation times and show very slight individual variation. Three plasmas were compared with each other with respect to the amount of heparin necessary to cause transition from complete coagulability to complete fluidity. The results of these comparisons are graphically portrayed in figure 1. In each case the optimum amount of calcium chloride for recalcification was found to be 0.1 cc. of a 2 per cent solution per cc. of plasma. After

addition of the desired quantity of heparin and 0.85 per cent saline solution to make a volume of 0.3 cc. in all tubes, 1 cc. of plasma was added, the samples were recalcified and the observations recorded at the end of one hour. At increments of 2 gamma of the standard sodium salt the inhibition of coagulation on recalcification was observed over a range of 4 to 60 gamma. It is apparent that recalcified sheep plasma is the best coagulation system for the quantitative measurement of the anticoagulant activity of unknown heparin preparations. An increase in heparin concentration of 2 gamma produced complete transition from coagulation to fluidity. A sheep plasma method for assaying heparin was therefore set up in which unknown preparations were compared to the standard which was always run in parallel.

The sheep plasma method of assay. Procedure: A series of standard tubes is set up containing 1.2, 1.4, 1.6, 1.8, 2.0 and 2.2 units of standard heparin with

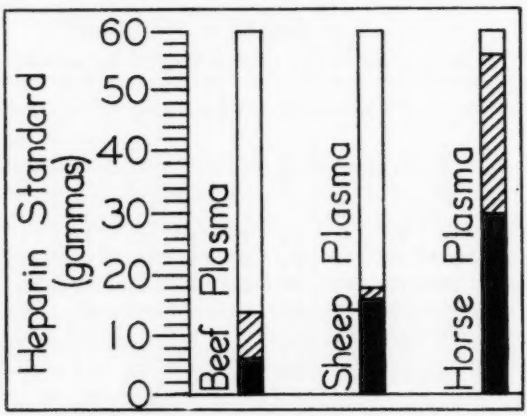

Fig. 1. The inhibition of coagulation of animal plasmas by addition of heparin is graphically portrayed in the figure. Complete fluidity is represented by the clear areas, complete coagulation by the totally black areas and partial coagulation by the cross lines.

the addition of 0.85 per cent sodium chloride solution so that the volume of solution in each tube is 0.3 cc. With most samples of sheep plasma the transition from clot to fluid will occur at the center of this range. It was seldom found necessary to change this range with different sheep plasmas. The addition of more tubes to include concentrations of 1 and 2.4 units may be found desirable. Similarly the unknown heparin solution is set up in a series of tubes covering a range of dosages estimated to be comparable to that of the standard. In case the approximate potency of the preparation to be tested is not known, it may be necessary to run a preliminary assay to determine the range. The volume of the heparin solution in each of the unknown tubes is made up to 0.3 cc. with 0.85 per cent NaCl solution.

One cubic centimeter of citrated sheep plasma is added to each of the tubes. In order that the time factor may be kept constant between standard and un-

known tubes, all series are arranged in ascending order of heparin content with the standard and unknown tubes in parallel rows. Working from left to right, the 2 per cent calcium chloride solution (usually 0.1 cc.) is added from a microburette alternately to standard and unknown tubes. As each solution is calcified, the tube is immediately stoppered with a paraffined cork and mixed by

TABLE 2

Assay of the sodium salt of heparin 185-LBS-1 and of the crude preparation 110-LBS-1

Stock solution of sodium Salt 185-LBS-1: 50 mgm. of powder dissolved in 0.85 per cent NaCl solution q.s. vol. = 50 cc. making 1 mgm./cc.

Stock solution of crude heparin 110-LBS-1: 50 mgm. of powder dissolved in 0.85 per cent NaCl solution, q.s. vol. = 50 cc. making 1 mgm./cc.

Standard heparin solution: 1000 units per cc.

	TUBE NO.	HEPARIN SOLUTION		0.85 PER CENT NaCl	AMOUNT OF HEPARIN	PLASMA	2 PER CENT $CaCl_2$	RESULTS 60 MINUTES
		Amount	Dil.					
		cc.		*cc.*	*units*	*cc.*	*cc.*	
Standard heparin, 1000 units per cc.	1	0.12	1–100	0.18	1.2	1	0.1	Cl
	2	0.14	1–100	0.16	1.4	1	0.1	Cl
	3	0.16	1–100	0.14	1.6	1	0.1	Cl
	4	0.18	1–100	0.12	1.8	1	0.1	Fl
	5	0.20	1–100	0.10	2.0	1	0.1	Fl
	6	0.22	1–100	0.08	2.2	1	0.1	Fl
					mgm.			
Heparin Na salt, 185-LBS-1, 1 mgm./cc.	7	0.12	1–10	0.18	0.012	1	0.1	Cl
	8	0.14	1–10	0.16	0.014	1	0.1	Cl
	9	0.16	1–10	0.14	0.016	1	0.1	Fl
	10	0.18	1–10	0.12	0.018	1	0.1	Fl
	11	0.20	1–10	0.10	0.020	1	0.1	Fl
	12	0.22	1–10	0.08	0.022	1	0.1	Fl
					mgm.			
Crude heparin, 110-LBS-1, 1 mgm./cc.	13	0.08		0.22	0.08	1	0.1	Cl
	14	0.10		0.20	0.10	1	0.1	Cl
	15	0.12		0.18	0.12	1	0.1	Fl?
	16	0.14		0.16	0.14	1	0.1	Fl
	17	0.16		0.14	0.16	1	0.1	Fl
	18	0.18		0.12	0.18	1	0.1	Fl

Calculations: At the end of sixty minutes tube no. 9 of the unknown Na salt series and tube no. 16 of the unknown crude heparin series matched tube no. 4 of the standard series. The Na salt contains therefore, 1.8 units in 0.016 mgm. or 112.5 units per mgm., while the crude heparin contains 1.8 units in 0.14 mgm. or 12.9 units per mgm.

inverting the tube three times. When not more than two unknown series are run in parallel with the standard, this operation requires approximately five minutes.

After recalcification the samples are allowed to stand at room temperature for one hour. At the end of this time they are examined for the extent of coagulation by inverting them, and the results recorded. With sheep plasma, usually

no questionable samples are encountered and never have we had more than one for different batches of sheep plasma. In each series the minimum amount of heparin necessary to prevent clotting is determined and the potency of the unknown solution is expressed in units of standard.

RESULTS. The procedure and calculations are illustrated in table 2 by a specific example in which the sodium salt 185-LBS-1 and the crude preparation 110-LBS-1 were assayed.

In order to compare the values obtained by the sheep plasma method and the cat method, two crude heparins, two barium salts and two purified sodium salts of heparin were tested by both methods. The results obtained using seven cats for each preparation agreed well with those obtained by the use of sheep plasma. The sheep plasma method has been used in controlling the fractionation of heparin from beef lung (8), yielding further evidence for its applicability to preparations showing wide difference in purity.

DISCUSSION. By application of the sheep plasma method here described, the anticoagulant activity of unknown heparin preparations can be quickly determined quantitatively. Since citrated sheep plasma has the property of forming a very firm clot in less than three minutes after recalcification, it is unnecessary to add a thromboplastin solution as MacIntosh (2) found necessary for horse plasma. When unknown heparin preparations are compared against the same standard the results obtained with sheep plasma are quantitatively comparable with those obtained by the cat method, provided several cats are used for each preparation. Thus, the principal advantage of the sheep plasma method lies in its simplicity and greater ease of operation. Comparative assays of the same preparations run independently by different individuals have yielded results which are reproducible within an error of not over 10 per cent.

SUMMARY

A method for assaying heparin has been described, based on the use of recalcified sheep plasma. Studies with horse, beef and sheep plasma indicated that sheep plasma is preferable because it gives a sharp end point with small increments in heparin dosage. The results obtained with the sheep plasma method are comparable to those obtained by the cat method, provided several cats are used. The sheep plasma method is simple, rapid, and yields reproducible results.

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TONE AND TENDON REFLEXES AFTER ASPHYXIATION OF THE SPINAL CORD[1]

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The course of recovery of reflex excitability after asphyxiating the spinal cord for various periods of time has been described before (1, 5). In the investigations reported in these papers, reflex activity was examined with only the usual clinical methods. For instance, extensor tone, which is the form of reflex activity returning most frequently after asphyxiation, was judged by the resistance of the leg against flexion. In the present study extensor tone and one of the tendon reflexes (kneejerk) were examined with graphic methods. This makes it possible to follow changes in the excitability of these reflexes more accurately, but makes it impracticable to extend the period of observation beyond 3 to 4 hours after cord asphyxiation. The changes in reflex excitability after that period are known from the aforementioned papers (1, 5).

METHODS. The spinal cord was asphyxiated as described before (1) by forcing Ringer solution in the isolated caudal part of the dural cavity under a pressure higher than the blood pressure. The femur was fixed on a board with screws, and the movements of the lower leg, elicited by contractions of the quadriceps muscle, were recorded by means of trolleys and a lever on a slowly revolving smoked drum. The quadriceps tendon was tapped with an electric hammer (3) at regular intervals. This tapping served the twofold purpose of stimulating the stretch receptors in the quadriceps, thus eliciting the kneejerk and enhancing extensor tone, and also of counteracting friction in the recording system and in the kneejoint which might mask slight changes in tone.

Action potentials, led off from the quadriceps with silver chloride plated silver wires, were amplified and recorded with a Mathews oscillograph.

Extensor tone and kneejerk after cord asphyxiation. Surprisingly soon after asphyxiating the spinal cord for periods up to 65 minutes, a slight and fleeting extensor tone was observed. The first sign of this "initial" tone became visible 4 to 16 minutes after asphyxiation was stopped. In a few minutes it reached a maximum, disappearing entirely 6 to 18 minutes after its beginning (table 1, fig. 1A and B). The initial tone never became high and though it could be found with certainty in the curves, its detection by examining the resistance of the leg against flexion is difficult. This tone has been observed after periods of asphyxiation of the cord ranging from 15 to 65 minutes; after longer asphyxiations (75 to 85 min.) no initial tone developed.

The initial tone was followed in all experiments by a period during which neither tone nor reflexes could be demonstrated (period of areflexia), then reflex

[1] This investigation was supported in part by a grant from the Paralysis Trust, Los Angeles.

activity started again. In the 65 and 55 minute experiments, a renewed extensor tone was usually the only activity observed. This "secondary" tone began 90 to 140 minutes after the end of asphyxiation (table 1). It reached a maximum

TABLE 1

All the figures, except those in the first column, indicate periods after the end of asphyxiation (minutes). The figures after the plus sign in the column marked "Tone on renewed asphyxiation" have the following meaning. In the 75 and 85 minute experiments the only sign of recovery from asphyxiation was the extensor tone on renewed asphyxiation. The presence of this phenomenon was tested every 20 to 30 minutes. The figures indicate when it was observed for the last time. The "R" in the seventh column indicates that secondary tone remains for the entire period of observation (3 to 4 hrs.).

DURATION OF ASPHYXIATION	INITIAL TONE		KNEEJERK BEGINNING	TONE ON RENEWED ASPHYXIATION	SECONDARY TONE	
	Start	End			Start	End
minutes	*minutes*	*minutes*	*minutes*		*minutes*	*minutes*
85				−		
85				−		
85				+ (90′)		
75				+ (60′)		
75				+ (140′)		
75				+ (120′)		
65				+	140	170
65	14	30		+ (180′)		
65	8	20		+	85	150
55				+	90	R
55	12	30		+	110	200
55	12	28	60	+		
45			130	+	210	R
45	16	22	65	+		
45	8	18	110	+		
35	6	12		−		
35	6	14	50	+		
35			150	+	100	160
25	10	24	90	+	120	R
25	6	18	50		55	130
25			80			
15	12	20	16			
15	6	14	31			
15	4	10	20			

in 15 to 30 minutes and disappeared again completely during the time of observation in three out of four experiments. The secondary tone after these long periods of asphyxiation is much stronger than the initial tone, so strong in fact

that the leg can often only be bent with difficulty. Figure 1A shows the relation between the height of initial and secondary tone. It is known from previous work (1) that after the disappearance of this tone usually no further signs of reflex excitability of the cord are observed during the rest of the animal's life. After the longest periods of asphyxiation (75 and 85 min.), no secondary tone developed.

After the shorter periods of asphyxiation (15 to 45 min., and in one of the 55 min. experiments), the first sign of reflex activity after the period of areflexia was the kneejerk which appeared 16 to 150 minutes after the end of asphyxiation

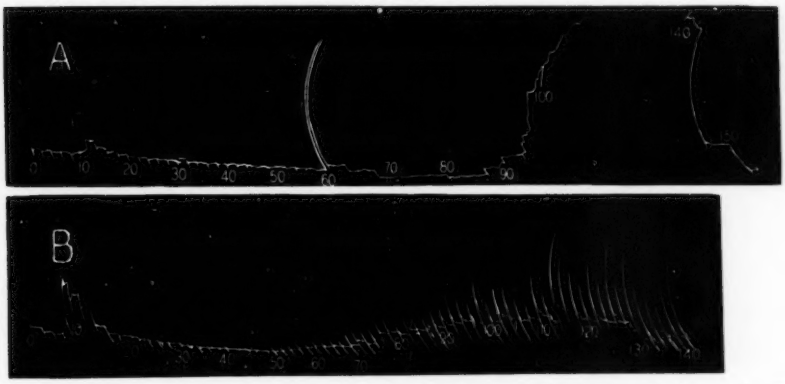

Fig. 1A. Course of quadriceps tone after a 65 minute asphyxiation of the cord. The quadriceps tendon is tapped every 2 minutes. The figures indicate the period after the end of asphyxiation in minutes. After about 8 minutes, initial tone develops which reaches a maximum after 12 to 14 minutes and disappears about 20 minutes after the end of asphyxiation. At 60 minutes the cord is subjected to a renewed asphyxiation of 20 seconds which results in strong extensor tone. After 85 minutes, secondary tone develops which, after reaching a maximum, disappears 150 minutes after the end of asphyxiation. The kymograph has been stopped for about 10 minutes at the maximum of secondary tone.

Fig. 1B. Tone and the kneejerk after 25 minutes of cord asphyxiation. The curve has to be read in the same way as 1A. Six minutes after the end of asphyxiation, initial tone develops which, after reaching a maximum, disappears 18 minutes after asphyxiation. About 50 minutes after the end of asphyxiation, a small kneejerk can be elicited which, after a few minutes, is followed by the development of a slight secondary tone. After about 130 minutes, this tone has disappeared again.

(table 1). After the 35 and 45 minute asphyxiations, this tendon reflex remained small, at least during the 3 to 4 hour period of observation. After the shorter periods of asphyxiation (15 and 25 min.) this reflex increased very quickly (fig. 1B).

In some of the 35 and 45 minute experiments the development of the kneejerk was followed by the appearance of secondary tone. In a previous paper (1) a very pronounced extensor tone has been described after asphyxiations of these durations. However, it usually took longer than a few hours for this strong tone to develop and it is likely that in a number of the present experiments the period

of observation has not been long enough for the secondary tone to appear. It was found previously (1) that this secondary tone was often temporary, like that in the 55 and 65 minute experiments; it not only took longer to develop, but it also remained longer (often 24–72 hrs.). In some experiments this high extensor tone remained for the rest of the animal's life.

In the 15 and 25 minute experiments the reflex excitability usually returned quickly to normalcy after the initial tone and period of areflexia. In the 25 minute experiments a slight and fleeting secondary tone developed before the reflexes became normal (fig. 1B).

The effects of renewed asphyxiation of the cord. During the period of areflexia, extensor tone can be elicited by renewed asphyxiation of the cord. This tone develops and disappears within the course of a minute. If the intradural pressure is not prolonged any more than necessary to demonstrate this phenomenon, it can be repeated each 20 to 30 minutes without apparent effect on the course of reflex excitability after asphyxiation. When the cord was asphyxiated shortly after the disappearance of the initial tone, the extensor tone developed after 20

Fig. 2. Extensor tone elicited by renewed asphyxiation during the period of areflexia 70 minutes after the end of a 55 minute cord asphyxiation. Asphyxia is caused by clamping the trachea (at *A*). The clamp is released and artificial respiration is begun after the extensor tone has disappeared (at *B*). The renewed oxygenation causes again a short period of extensor tone. Respiration is visible as small spikes in the curve. Time in seconds.

to 25 seconds and was not very strong. At the end of the period of areflexia, however, asphyxiation caused a very strong extensor tone which sometimes started as soon as 5 seconds after the renewed asphyxiation. The tone reached a maximum in a few seconds and disappeared 15 to 20 seconds after its beginning. Renewed asphyxiation had no effect before the development of the initial tone and after the disappearance of the secondary tone (in the 55 and 65 min. experiments). In all of the 75 minute and in one of the 85 minute experiments extensor tone was elicited by renewed asphyxiation of the cord, though neither initial nor secondary tone develops after these long periods of asphyxiation. The renewed asphyxiation was effective only for a limited period, which corresponded roughly with the period of areflexia in the 65 minute experiments (table 1).

In some of the experiments renewed oxygenation of the cord, by release of the short lasting pressure in the dural cavity, elicited a new period of tone. This is illustrated in the curve of figure 2 in which asphyxiation of the cord was effected by clamping the trachea instead of by forcing Ringer solution into the dural cavity.

The nature of the tone observed. A. *Secondary tone.* It has been found pre-

viously (1) that the extensor tone after asphyxiation, which now can be identified as secondary tone, can be decreased and sometimes even abolished by cutting the dorsal roots relevant to the muscles examined. From this result the conclusion was drawn that this tone is a reflex tone. The following study of the action potentials of the quadriceps muscle during the period of secondary tone supports this conclusion.

With the first signs of secondary tone, small spikes of low frequency became visible in the electrogram. As the tone developed the frequency and size of the action potentials increased until at the maximum of tone large spikes of high frequency were led off from the muscle. When, as in the 55 and 65 minute experiments, the secondary tone disappeared again, the electrical activity diminished and finally disappeared.

With respect to the all-or-none relation, the growth of the spikes, as tone develops, can hardly be caused by a growth of the action potentials of the individual motor units, but is probably caused by an increase in the number of active units. When the tone is slight, only a few motor units will be active and the chances are that none of them is located in the immediate neighborhood of the leading-off electrodes. This accounts for the small action potentials usually found at this stage. As the tone increases, more units become active and the chances that one or more will be situated near the leading off electrodes become greater, resulting in an apparent growth of the action potentials.

The electrical activity during secondary tone can be influenced by changing the length of the quadriceps muscle. During the first development of secondary tone, when hardly any electrical activity was present, spikes could be elicited by flexion of the knee, which stretches the quadriceps (fig. 3A). During a period of moderate tone and electrical activity, the frequency and size of the spikes could be greatly increased by the same stretch stimulus (fig. 3B). When in such experiments the stretch was stopped, a silent period occurred after which the moderate activity was resumed. Also, it was observed that during a period of considerable electrical activity, a relaxation of the muscle by extending the knee resulted in a temporary arrest of all action potentials (fig. 3C).

These reactions of the action potentials on stretch and relaxation definitely characterize the secondary tone as a reflex tone, elicited by impulses from the stretch receptors in the muscle.

Sometimes at the maximum of secondary tone, accompanied by great electrical activity, the influence of stretch and relaxation could not be demonstrated. It is likely that when the reflex activity of the cord is very high, other impulses than those elicited in the stretch receptors can reach the quadriceps motor neurons. Under these circumstances, little effect of changes in the muscle length can be expected.

B. *Initial tone.* Since the interval between the end of asphyxiation and the beginning of the initial tone is too short to sever the dorsal roots, this method cannot be used to establish the nature of this tone. The action potentials of the initial tone, however, can be investigated.

With the first signs of initial tone, spikes appear in the electrogram which

grow in size and frequency with the increase of tone. A maximum is reached in a few minutes which is less pronounced than that during the maximum of secondary tone. Then tone and action potentials decrease and disappear entirely.

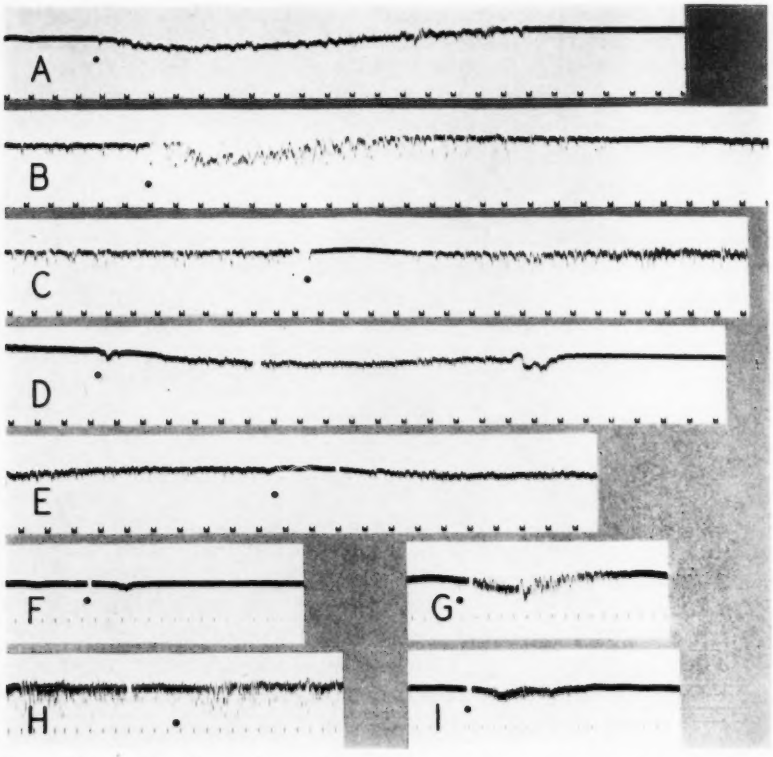

Fig. 3. Effect of bending and stretching the knee on the action potentials of the quadriceps muscle during secondary and initial tone and during the extensor tone caused by renewed cord asphyxiation in the period of areflexia. In each of the curves the beginning of flexion or extension of the knee is indicated by a dot.

A. Seventy minutes after a 50 minute asphyxiation of the cord, when secondary tone begins to develop, flexion of the knee causes considerable electrical activity. B. Twenty minutes later spontaneous activity is greatly enhanced by bending the knee. This increased activity is followed by a silent period. C. Stretching of the knee stops spontaneous electrical activity. This curve is taken a few minutes after B.

D. Eight minutes after a 50 minute cord asphyxiation, when initial tone starts to develop, flexion of the knee causes electrical activity. E. After 10 minutes, continuous electrical activity is present which can be stopped by stretching the knee.

F, G, H, I. During the period of areflexia, 110 minutes after a 50 minute cord asphyxiation, the spinal cord is subjected to renewed asphyxiation. F. Before asphyxiation flexion of the knee has the slightest effect on the electrogram. G, H, I are taken respectively 10, 18 and 30 seconds after the beginning of renewed asphyxiation. G and I show clearly the effect of bending the knee. H shows the great electrical activity at the height of tone. Knee bending has only little effect at that time.

The same influence of stretching (fig. 3D) and relaxation (fig. 3E) as described for the action potentials of the secondary tone has been observed in the electrogram of the initial tone. The initial tone thus can be considered as a reflex tone caused by impulses from the stretch receptors in the muscle.

C. *Tone caused by renewed cord asphyxiation.* The electrogram of the quadriceps during the period between initial and secondary tone did not show any activity. However, at the end of this period stretch of the quadriceps caused in some animals a few small spikes. The strong tone elicited by renewed cord asphyxiation during the period of areflexia was accompanied by the development and subsequent disappearance of large action potentials of high frequency. In some experiments a distinct influence of stretching the quadriceps muscle could be found during the renewed asphyxiation. This is shown in the curves F, G, H, I, of figure 3. Before the renewed asphyxiation, the stretch stimulus had a just visible effect (fig. 3F). This effect had greatly increased 10 seconds after the beginning of the renewed asphyxiation (fig. 3G). The maximum of electrical activity was reached in about 18 seconds after asphyxiation. Stretching the muscle had only little effect at this stage (fig. 3H). After the decline of the action potentials, however, stretch of the muscle again elicited spikes (fig. 3I taken 30 sec. after the beginning of asphyxia). In many experiments the influence of stretch on the action potentials has been less distinct. It can be concluded, however, that the tone elicited by renewed asphyxiation also has the character of a reflex tone.

Tone during renewed cord asphyxiation was not prevented by transection of the dorsal roots supplying the leg. In these experiments the renewed asphyxiation had to be carried out by clamping the trachea since the spinal canal had been opened to sever the roots. It is likely that when the reflex excitability of the cord becomes high enough, the sensory influx from the heterolateral side and from other segment levels is sufficient to produce quadriceps contraction.

Discussion. One of the features of the reflex activity after 55 and 65 minute asphyxiation is that it returns temporarily (1). After the often high secondary tone has disappeared, no reflexes or pronounced tone are observed during the rest of the animal's life. This temporary return of reflex activity has been explained by the assumption that the nervous elements, though being able to function after the asphyxiation, are damaged so severely that they will eventually be totally destroyed. This view is substantiated by metabolic and histologic studies of the cord at various intervals after a 60 minute asphyxiation (2). The first hours after asphyxiation the metabolism of the cord was of almost normal intensity, and the nerve cells showed a normal picture. After a few hours, however, the metabolism decreased and the nerve cells showed changes which, in the next 48 hours, resulted in their total disappearance.

The great height of the secondary tone has been explained by the assumption that the inhibitory structures in the cord are more readily damaged by asphyxiation than the excitatory ones (1). This would result in a disbalance of their respective functions and thus cause an increased reflex excitability of the cord manifest in the high extensor tone. The conception of the high secondary tone as a release phenomenon was based on the observation that it sometimes (es-

pecially after 35 min. asphyxiation) remained for the rest of the animal's life (several weeks).

The following explanations are offered for the course of tone after asphyxiation. The presence of an initial reflex tone 4 to 16 minutes after the end of a 55 to 65 minute asphyxiation shows that synaptic conduction recovers very quickly. It is hardly likely that synaptic conduction, once it has recovered, becomes impossible during the period of areflexia to awaken again during the secondary tone. It is much more likely that synaptic conduction is potentially possible from the beginning of the initial tone until the end of secondary tone, but is depressed during the period of areflexia. This depression may be due to the recovery of inhibitory structures in the cord. The appearance and disappearance of initial tone then is caused by a slight difference in the time of recovery of the excitatory and inhibitory structures in the cord. Thus for a few minutes the excitatory structures are unopposed by inhibition, which results in initial tone. During the period of areflexia the inhibitory structures become so active that a suppression of all reflex activity results. This explanation of the period of areflexia is supported by the observation that reflex activity can be reestablished during this period by renewed asphyxiation of the cord. The renewed asphyxiation would depress the activity of the inhibitory structures before abolishing synaptic conduction, resulting in reflex tone of short duration. When the cord recovers from the renewed asphyxiation, synaptic conduction may recover before the functioning of the inhibitory structures, resulting also in a short period of tone (fig. 2).

The development of secondary tone begins when the inhibitory structures in the cord, which are more sensitive to oxygen lack, are being destroyed as a belated effect of the asphyxiation, thereby leaving the excitatory functions unopposed. This results in the high secondary tone. When a short while later the excitatory structures also are destroyed the secondary tone disappears, not to return for the rest of the animal's life.

A similar explanation can be given for the course of reflex excitability after 45 and 35 minutes' asphyxiation.

It is possible, however, that the depression of the reflex excitability between initial and secondary tone is not due to a recovery of the inhibitory structures in the cord, but is caused by a depression in the excitability of the asphyxiated nervous elements. This possibility is suggested by Lehmann's (4) observation of changes in excitability of excised peripheral nerves after asphyxiation. He found that after a 15 minute asphyxiation the nerve excitability, after an initial recovery, decreases again for a period of about 30 minutes. If it is assumed that similar changes in excitability develop under the conditions of the present experiments in the nervous elements in the cord, this could account for the depression reflex excitability between initial and secondary tone. An explanation of the tone on renewed asphyxiation of the cord during the period of areflexia is, in view of these considerations, not directly obvious.

I am indebted to Mrs. J. Wiersma, Mr. C. H. Ellis and Mr. E. B. Wright for valuable assistance.

SUMMARY

1. Five to 15 minutes after the end of a 15 to 65 minute asphyxiation of the spinal cord, a slight extensor tone develops which, after reaching a maximum, disappears within 30 minutes after asphyxiation (initial tone).

2. The initial tone is followed by a period in which neither tone nor other forms of reflex activity are present (period of areflexia). Extensor tone can be elicited during this period by renewed asphyxiation of the cord.

3. In the 65 and 55 minute experiments, a high extensor tone develops after the period of areflexia. This secondary tone is temporary and disappears after a few hours. In the 45 and 35 minute experiments, tendon reflexes develop after the period of areflexia, followed by secondary tone. The secondary tone is usually temporary in these experiments also, but sometimes remains for the rest of the animal's life. In the 25 and 15 minute experiments, the period of areflexia is followed by the development of tendon reflexes and usually the return to normal reflex excitability.

4. It has been possible to prove the reflex nature of initial and secondary tone and of the extensor tone elicited during the period of areflexia by renewed asphyxiation of the cord.

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THE PHYSIOLOGICAL RESPONSE TO ENTEROCRININ CONSIDERED QUANTITATIVELY[1]

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Methods for preparing enterocrinin and roughly estimating the amount present in intestinal extracts have been described in a previous paper (Nasset, 1938). The very erratic behaviour of the intestine is quite commonly known among those who have worked with it. The secretory response to intravenous injections of the intestinal hormone, enterocrinin, is no exception; indeed the variability is such as to make very difficult the quantitative determination of enterocrinin potency by any of the types of assay procedures ordinarily used for quick acting hormones and drugs. Since the availability of an accurate and reasonably rapid method of assay is extremely important in any attempt to isolate a physiologically active substance, the intestinal response to enterocrinin was subjected to a rather exhaustive study. The method of assay developed from this study and described below does not differ in fundamental principles from that described earlier, but various refinements in the procedure have greatly increased the accuracy obtainable with a limited number of assays.

METHODS. Anesthetized[3] and dewormed dogs weighing about 20 kgm. were used for all assays. Two adjacent 15 cm. loops of the jejunum were isolated just below the ligament of Treitz and flushed out with warm isotonic saline. Close-wound aluminum wire coils with glass cannulae taped to both ends were inserted as gently as possible and the loops securely tied to the taped ends of the coils. The loops, supported outside of the abdomen, were maintained in good condition with a moist chamber similar to that described by Nasset and Parry (1934).

A thermocouple was inserted into the lumen of one loop through a T-cannula, one end of each loop connected to a drain flask, and the other end connected to reservoir and drop recorder system such as that shown in figure 1. All air was flushed out of the loops by warm saline from reservoir *C*, the drain tubes closed, reservoir *D* filled with saline, and *A* and *D* connected. The reservoir system was lowered until the dropper *E* was about 10 cm. below the level of the loops and allowed at least thirty minutes to come to equilibrium. The platinum foil electrodes *F* were placed in series with a 100-watt lamp and an ordinary kymograph signal magnet in a 110 volt D.C. circuit to function as a very simple but extremely efficient drop recorder capable of recording almost any rate of flow short of a steady stream.

[1] This investigation was supported in part by grants from the Parke, Davis Co. and the Committee for Research in Endocrinology of the National Research Council.

[2] The data in this paper are taken from a thesis presented by R. M. Fink to the Graduate School, University of Rochester, in partial fulfillment of the requirements for the degree, Doctor of Philosophy.

[3] Dial-Urethane, kindly supplied by the Ciba Pharmaceutical Co.

The carotid artery was cannulated and connected to a recording blood pressure manometer. A femoral vein was cannulated and attached to a saline-filled burette to facilitate the injection of hormone preparations.

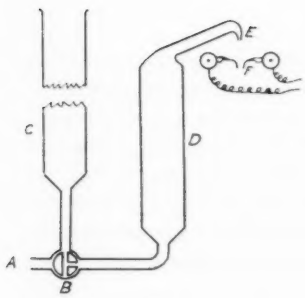

Fig. 1

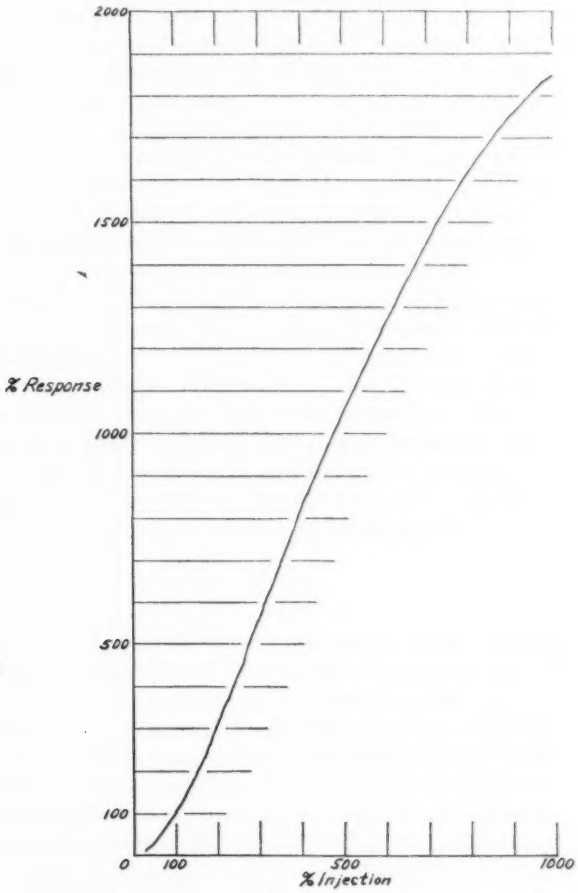

Fig. 2

Fig. 1. *A*—connection to loop; *B*—3-way stopcock, 4 mm. bore; *C*—200 ml. saline reservoir; *D*—75 ml. reservoir; *E*—dropper, delivers 33 to 34 drops/ml.; *F*—heavy platinum foil electrodes.

Fig. 2. Curve of response to enterocrinin

If it was desired to estimate the amount of secretin in the preparations to be assayed, the major pancreatic duct was cannulated and attached to a drop recorder before the intestinal loops were isolated.

The temperature of the abdominal chamber was adjusted by means of a rheostat to maintain a loop temperature of about 38°C.

The level of the droppers was adjusted until a low rate of apparent secretion (2 drops/min., or less) was obtained from both loops (the two droppers being always moved together, so that the loops were under equal negative pressures). After allowing the loops about five minutes to adjust themselves to the new pressure, the kymograph was started and this basal, or control, rate recorded for at least five minutes. An injection of a standard enterocrinin preparation was then made and the kymograph recording continued for at least five minutes longer than the period, p, required for the rate of secretion to return to a basal rate in both loops (usually 15 min. was sufficient). An amount of an unknown preparation estimated to be equivalent to the standard injection was then administered. After the secretory rate had again returned to a basal rate and remained there for at least five minutes, a second standard injection was made and the recording continued as before. This group of three injections comprised the basic assay unit. The number of drops in the five-minute period preceding each injection was recorded as a control, C, value, and the number of drops obtained between the time of an injection and the beginning of the next control period was recorded as total, T, value. Each unit assay, then, involved the recording of 7 values for each of the two loops, as follows: 1, control; 2, total for standard; 3, control; 4, total for "unknown"; 5, control; 6, total for standard; 7, control. When a T value referred to a ten minute period, the sum of the previous and the subsequent C values was subtracted from it in order to determine the net response, N, to the injection. When the period was greater than ten minutes the sum of the C values was multiplied by an appropriate factor before subtracting. That is:

$$N = T - (C_1 + C_2)\frac{p}{10}$$

where N is the net response to an injection, T is the total number of drops in the period following the injection, C_1 and C_2 are the previous and subsequent five-minute control values, and p is the period (in minutes) used in determining T. The net response to the unknown preparation was then divided by the average of the net responses to the previous and subsequent standard injections, and the quotient multiplied by 100 to give the response to the unknown as a percentage of the standard responses used, giving each standard a weight in inverse proportion to the interval between it and the unknown injection, as follows:

$$R = \frac{100\,Nu\,(Z_1 + Z_2)}{Ns_1 Z_2 + Ns_2 Z_1}$$

where R is the per cent response of the unknown in terms of the standard, Nu, Ns_1 and Ns_2 are the net responses to the unknown and the previous and subsequent standards, respectively, Z_1 is the time interval between the injection of the previous standard and the injection of the unknown, and Z_2 is the interval between the injection of the unknown and the injection of the subsequent standard.

This method of comparing two preparations is called the "double control, double standard" method, and it, more than any other single factor, is to be

credited with making the highly variable intestinal response yield accurate and reproducible assay results. The method estimates the "expected" control value during the period in which an injection is acting, and the "expected" standard response during the period in which an "unknown" injection is acting, by assuming that the basal rate and the responsiveness to enterocrinin remain constant or increase or decrease at a uniform rate over the periods involved. In a number of tests, the assumption has been found to be sufficiently valid to make the "double control, double standard" method of comparison highly superior to any other method tried.

If the average net response to the standard $\frac{Ns_1 + Ns_2}{2}$ is approximately 10 drops, the enterocrinin curve of response (fig. 2) is entered through the ordinate with the value of R as determined above, and the corresponding per cent injection (I) value read off on the abscissa. This figure is an estimate of the amount of enterocrinin in the dose of unknown expressed as a percentage of that present in the standard dose. When the average net response to the standard differs considerably from 10 drops, the curve of response is entered twice, once at the ordinate equal to ten times the average net response to the standard, and again at the ordinate equal to ten times the net response to the unknown injection ($10\ Nu$). The corresponding abscissa values in this case relate both the standard and the unknown to the point (100, 100) on the curve, and the value of I is found by dividing the abscissa value for the unknown by that for the standard and multiplying by 100. Since a large portion of the central part of the curve closely approximates a straight line this method is used only when the standard response is very large or very small.

The curve of response is an average constructed from data collected during routine enterocrinin assays on more than fifty dogs. All series of graded doses of a single preparation given to the same dog in a short time were assembled, the response in each series nearest to ten drops (0.3 cc.) was taken as 100 per cent response, and the corresponding dose taken as 100 per cent injection. The rest of the values in each series were expressed in terms of this fixed point, and in this manner over 300 points were obtained for the curve, about 100 of which were arbitrarily fixed at the point (100, 100). The extremities of the average curve may differ widely from the curves of response for individual dogs, but the variation becomes much less as the point (100, 100) is approached since all individual curves meet at that point and vary only in slope and linearity. In order to facilitate reproduction, the following empirical equations for the average curve were derived:

(A) $$R = (0.191\ I - 0.9)^{1.59}$$

(B) $$R = 0.1418\ I^{\frac{1}{0.565+9.34I\cdot 10^{-5}}}$$

(A) gives a very good fit from the point (35, 15) to the point (200, 315), while (B) gives a fairly good fit over the whole range up to the point (1000, 1840). Obviously, no physiological significance can be attached to such equations.

The per cent potency, P, of the unknown as compared with the standard was calculated by averaging the I values as determined for the two loops, dividing this average by the dose of unknown in milligrams (Du) and multiplying by the dose of standard in milligrams (Ds); i.e.,

$$P = \frac{(I_u + I_1)D_s}{2\,Du}$$

where I_u and I_1 refer to the I values from the upper and lower jejunal loops, respectively.

Either the average I values or P was used to calculate a dose of unknown which should equal the activity of the standard dose. If the responses were still considerably different in the second assay of the unknown, the results of this assay were used to calculate a third dose of unknown, and so on until two values of P checked within ± 10 per cent. Ordinarily, such a check may be obtained with three assays of a previously untested preparation.

When a number of unknown preparations are to be assayed against the same standard, the unknowns are assayed alternately in order to permit the assays to proceed while subsequent doses are being calculated. In this way n assays may be carried out using $n + 1$ doses of the standard and usually at the rate of about two assays per hour. The dose of standard may be varied to keep the response within workable limits without breaking the chain of alternate standard and unknown injections. In this case P is calculated from the previous and subsequent standard responses individually and the values so obtained are then averaged.

Inasmuch as enterocrinin has not as yet been prepared in a pure state and no large stock of a stable standard preparation is available for distribution, it was desired to relate the expression of potency to the absolute value of the physiological response. This was accomplished by setting one preparation aside as a "permanent standard" and testing it occasionally in the animals used for routine assays. After each test the average curve of response was used in estimating the amount of the preparation required to elicit the "threshold response" of 10 drops (0.3 cc.) over the basal rate (calculated from the previous and subsequent control values, as described above). The mean "threshold dose" ($T.D.$) of the "permanent standard", as determined from 42 such tests in 11 dogs, was 52 mgm., with the probable error of the mean ($P.E_M.$) being $\pm$ 4 mgm. The preparation showed no evidence of instability during the 12-month period covered by the tests. The stability was further verified by the fact that the coefficient of correlation, r, between the various values of the $T.D.$, and the age of the preparation at the time the values were determined, was only $+0.02$, $P.E._r$ ± 0.10. Any serious decrease in potency should have resulted in a significant, positive value of r. The preparation was stored over $CaCl_2$ at atmospheric pressure. Highly purified preparations were found to be much less stable.

Unknown preparations were assayed against the permanent standard, and their threshold doses determined as follows:

$$T.D._u = \frac{T.D.s \times P}{100}$$

where *T.D.u* and *T.D.s* are the threshold doses of the unknown and the standard, respectively. These preparations could then be used as temporary, or assay, standards for assigning *T.D.* values to other preparations.

The question as to whether the dose should be expressed in terms of the animal's body weight (e.g., as mgm./kgm.) was settled statistically as shown in table 1. The statistics shown involve all the data available on one preparation. The term dose · Wt.$^{-1.0}$ refers to the dose expressed as mgm./kgm. of body weight, while the term dose · Wt.$^{0.0}$ refers to the dose expressed in milligrams, without reference to body weight. The weight enters to varying degrees in the other terms. Inasmuch as the odds are about 14,000 to 1 against a difference such as that between the first and fourth r values occurring by chance (P = 0.00007), the expression of the dose without reference to body weight is clearly preferable. The correlation ratios show the same trend as the correlation coefficients, and incidentally prove that the curve of response to enterocrinin is significantly non-linear. Analyses similar to the above showed that the dose of secretin is likewise best expressed without reference to body weight, while the dose

TABLE 1

Correlation of the net response with the dose expressed in various ways

(Number of paired observations, 160; number of dogs, 9; weight range, 13 to 30 kgm.)

X	Y	r_{xy}
Net (drops)	Dose · Wt.$^{-1.0}$	+0.24
Net (drops)	Dose · Wt.$^{-.67}$	+0.34
Net (drops)	Dose · Wt.$^{-.33}$	+0.42
Net (drops)	Dose · Wt.$^{0.0}$	+0.45
Net (drops)	Dose · Wt.$^{+.33}$	+0.38

of the vasodilatins present in intestinal extracts should be related to body weight (e.g., as mgm./kgm.).

Discussion. The highly corrected and controlled method of assay described above was made necessary by the high degree of variability of the intestinal secretory response. The description of the method seems rather involved, but in practice it is really quite workable, requiring little more time and material than a number of less precise assay schemes in use.

The "double control, double standard" procedure has been found quite useful in the assay of secretin and vasodilatins, though in these instances it is not so important as in the case of enterocrinin, for the variations in responsiveness are neither so marked nor so rapid. The method is believed to be worthy of consideration for many procedures where period to period variations in responsiveness are encountered.

Although the statistical analysis showed that it was better not to base the dose of enterocrinin upon the weight of the dog being used, certain theoretical considerations make it appear probable that the response to a given amount of a preparation is not completely unrelated to the body weight, and it is for this reason that a reasonable effort is made to keep the weight near a standard value

of about 20 kgm. This question is purely academic as far as accurate comparison of standard and unknown preparations is concerned, but it is important in establishing absolute, or permanent standards.

Vasodilatins have relatively little effect upon the assay results. The secretory response to a powerful vasodilatin injection comes almost immediately as a short spurt of secretion and is followed by a period in which the basal rate is reduced, so that these two periods nearly cancel. For very accurate assays of strongly vasodilatory preparations, the enterocrinin part of the preparation may be inactivated by treatment with alkali, injected before and after the injection of the active preparation, and the previous and subsequent periods treated as control values.

The parallelism of enzyme and volume responses reported by Nasset and Pierce (1935) has been confirmed using highly potent enterocrinin preparations. The enzyme response tends to return to a basal level more slowly than the volume response, particularly when large doses are given. In one series that was analyzed statistically, the coefficient of correlation between the volume and the invertase responses was +0.84, between volume and phosphatase +0.82, and between invertase and phosphatase +0.99.

SUMMARY

A detailed procedure is given for the bioassay of enterocrinin, the hormone which excites the secretory glands of the intestine. The principal feature of the method, which permits accurate and reproducible results to be obtained quickly from the highly variable intestinal secretory response, is its precise treatment of the time factor in the normal variations in responsiveness. The general procedure outlined may well be of value in increasing the accuracy and practicability of other types of bioassays.

An average curve of response to enterocrinin is used to assist in equilibrating the responses to the standard and the "unknown" preparations.

A statistical analysis of data on 9 dogs shows a closer dose: response correlation when the dose of enterocrinin is given without reference to the weight of the dog than when it is given on a *per kilo weight* basis. Secretin, likewise, was found to give more uniform responses in different dogs if the same dose were given, regardless of body weight, while the vasodilatins present in intestinal extracts are best expressed on a *per kilo* basis.

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THE FRACTIONATION OF ENTEROCRININ PREPARATIONS[1]

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Methods of preparing enterocrinin and testing for its presence in intestinal extracts have been described in a previous paper (Nasset, 1938). A description of an accurate and rapid method for assaying the hormone is given in the accompanying paper. Since there have been some changes made in the basic fractionation procedure along with the addition of new steps, the improved methods that are now in use will be described in full.

The small intestines of hogs (or dogs or cattle) were removed immediately after death of the animals, turned inside out, washed rapidly in water, and extracted with 87 per cent alcohol containing 3.3 cc. of concentrated HCl per liter (3 l./kgm. of tissue). The extraction was continued for 72 hours, with occasional stirring, at about 5°C. The alcohol solution (fraction 2) was decanted, allowed to settle, and the supernatant concentrated to $\frac{1}{6}$ its volume under reduced pressure at about 40°C. (A "foam-head still" was a great help in this step.) NaCl (300 grams/liter) was dissolved in the crude water solution (fraction 4) while it was still warm, and the mixture set in the cold room overnight. The precipitate was filtered off and removed from the filter while still damp. It was then pressed or pounded between successive layers of filter paper, until no more moisture could be expressed. (This step usually removed the major portion of the vasodilatins.) The precipitate was further dried in vacuo. The dry preparation was shaken with glacial acetic acid (10 cc./gram) for about three hours and then with 5 cc./gram for about one hour. The preparation was precipitated from the acetic acid solution by addition of 4 volumes of an ether mixture (3 parts ethyl, 1 part petroleum), washed twice with ether and once with acetone and dried *in vacuo*. The dry product (fraction 10) was extracted twice with acetic acid (15 cc./gram) and reprecipitated, washed, and dried as above. The dry, white powder (fraction 14) was shaken for several hours with methyl cellosolve (0.07 cc./mgm.), one-half volume of ethyl ether added very slowly with stirring, the mixture covered to prevent evaporation, allowed to stand about an hour at room temperature, and then centrifuged. The precipitate was discarded and to the supernatant was added $2\frac{1}{2}$ volumes of ethyl ether and one volume of petroleum ether (both based on the original volume of methyl cellosolve used). The mixture was allowed to stand several hours in the cold, centrifuged, and the precipitate (fraction 18) washed and dried as above. Fraction 18 was dissolved

[1] This investigation was supported in part by grants from the Parke, Davis Co. and the Committee for Research in Endocrinology of the National Research Council.

[2] The data in this paper are taken from a thesis presented by R. M. Fink to the Graduate School, University of Rochester, in partial fulfillment of the requirements for the degree, Doctor of Philosophy.

in propylene glycol (0.1 cc./mgm.), 8 to 10 volumes of acetone added slowly, with stirring, placed in the cold room overnight, centrifuged, and the precipitate (fraction 20) washed three times with acetone and dried *in vacuo*. The fractionation from acetic acid, methyl cellosolve, and propylene glycol may be repeated, if desired, with little loss of active material. Fraction 20 was dissolved in water (0.02 cc./mgm.), and $1\frac{1}{2}$ vols. of a saturated water solution of picric acid added slowly. The mixture was set in the cold room for 24 to 72 hours and then centrifuged. The precipitate was shaken with two volumes of acetone (this and all further volumes to be based on the original volume of water used with fraction 20), the insoluble residue dried *in vacuo*, and then extracted three times with two-volume portions of hot water (about 90°C.). The combined water extracts were evaporated to dryness *in vacuo*, the residue extracted with two volumes of warm glacial acetic acid (about 60°C.), the insoluble residue discarded, and the solution allowed to cool to about room temperature. The yellow picrate may be reprecipitated at this point by addition of ether, but there is usually little advantage in doing so. HCl gas was bubbled through the acetic acid solution of the picrate until the yellow color disappeared, and eight volumes of ether added. The mixture was set in the cold for several hours, centrifuged, and the precipitate (fraction 30) washed with ether containing HCl gas, with ether alone, and with acetone. It was then dried *in vacuo* and stored over $CaCl_2$ either *in vacuo* or in an atmosphere of nitrogen.

In general, each of these steps was first thoroughly studied individually and then worked into the regular procedure at the most suitable point, conditions being selected with an eye to maintaining a reasonably good recovery of the enterocrinin during the process of purification.

The products obtained by the procedure as outlined above are not strictly uniform, tenfold differences in potency being not uncommon. Aside from the variability in the raw material, the salting-out procedure is the most wasteful and unreliable step, but no other procedure or combination of procedures has been found which will satisfactorily carry out its functions.

Figure 1 shows the assay results for the principal fractions of a dog gut preparation. It will be noted that up to fraction 14 the procedure was a better preparation of secretin and vasodilatin than of enterocrinin. The last three steps, however, greatly increased the enterocrinin potency, with relatively small losses, and at the same time removed all but 1 per cent of the secretin and reduced the vasodilatin content to a point where it was not detectable even with doses many times those required for the enterocrinin assay. The threshold dose[3] of the preparation was reduced from 12 mgm. (fraction 2) to 36 micrograms (fraction 30).

In addition to the data given, all the discarded fractions were worked up and assayed, and about 80 per cent of the original enterocrinin activity accounted for. In this preparation about 70 per cent of the enterocrinin was lost during the

[3] The threshold dose of an enterocrinin preparation may be defined as the dose required to elicit 10 drops (0.3 cc.) of extra intestinal secretion from an *average* dog under strictly specified conditions. (See accompanying paper.)

salting-out step while comparatively little secretin or vasodilatin was removed. In the next dog gut preparation, however, this step lost about the same amount of enterocrinin, but removed 98 per cent of the secretin and all the vasodilatins. The usual result lies somewhere between these two extremes. Similar variation is encountered in hog gut preparations, but there the final potency achieved is

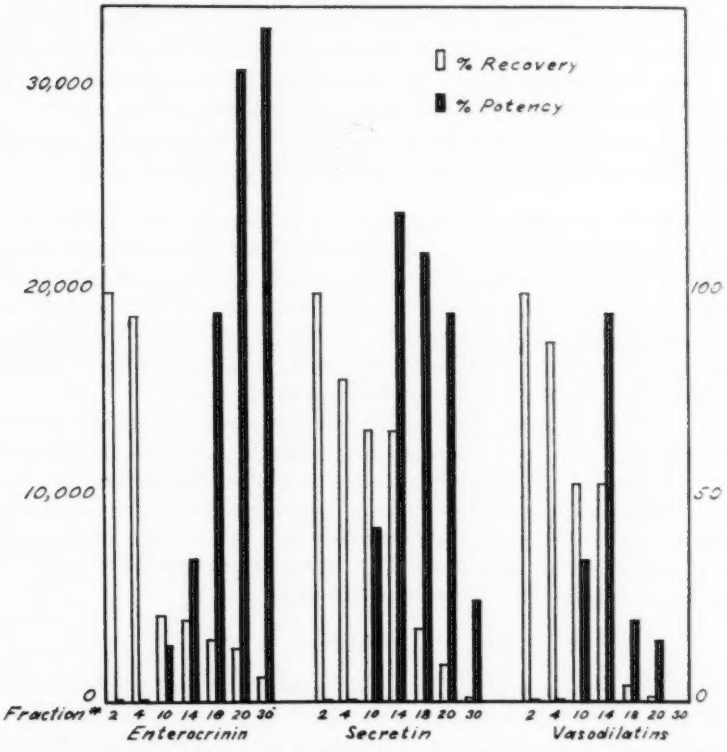

Fig. 1. Fractionation of an enterocrinin preparation. The open bars and the figures at the right indicate the percentage recovery in each of the fractions, of the three physiologically active substances named at the bottom of the groups, with the amount of each in fraction 2 taken as 100 per cent. The solid bars and the figures at the left represent the per cent potency of the three substances in each fraction, with their potency in fraction 2 taken as 100 per cent. The numeral below each pair of bars refers to the number of the fraction as given in the description of the fractionation procedure.

only about $\frac{1}{5}$ to $\frac{1}{10}$ of that obtainable from dog gut and the weight of the final fraction is proportionately larger (about 100 to 500 mgm. from 20 hog small intestines).

A number of other fractionation procedures have shown promise but have not been sufficiently studied to justify their incorporation into the regular procedure. Among this group are dialysis, electrophoresis, chromatographic adsorption, and

a number of precipitating agents. The most interesting results with dialysis experiments were obtained while using a cellophane membrane with aqueous acetic acid or aqueous methyl cellosolve as solvents. Under these conditions the porosity of the membrane may be controlled by adjusting the amount of water in the mixed solvent. Enterocrinin and secretin dialyzed at approximately the same rate under all conditions tested. In water solution most preparations are almost completely dialyzable, even through thick parchment. In the electrophoresis of crude extracts the enterocrinin moves toward the cathode ahead of the bulk of the preparation. The picrate derivative would appear to be the most suitable substance for chromatographic adsorption studies.

Attempts to purify enterocrinin by trichloracetic acid precipitation, isoelectric precipitation, electrodialysis[4], and molecular distillation have yielded very poor results.

The stability of enterocrinin has been found to vary widely according to the purity of the preparation used. In moderately pure preparations enterocrinin is relatively stable with respect to dilute acids and bases, heat, and oxidation. Very highly purified preparations, however, have lost a substantial proportion of their activity on being left open to the air in a $CaCl_2$ desiccator for a few months, and water solutions of these very potent preparations deteriorate within a few days or weeks even while stoppered and stored at 5°C.

No estimate can be given of the activity of pure enterocrinin, but it is certainly a powerful substance and a considerable amount of progress has been made toward its isolation. A check of the assay data concerning the most active preparation reported by Nasset (1938), using insofar as possible the new, improved methods of calculation, showed its threshold dose to be about 800 micrograms, as compared to the 80 microgram T.D. reported by Fink and Nasset (1941) and the 36 microgram T.D. reported in this paper.

SUMMARY

A detailed procedure is given for obtaining highly potent preparations of enterocrinin, the hormone which excites the secretory glands of the intestine. The procedure involves the extraction of intestines with slightly acidic 87 per cent alcohol, removal of the alcohol, salting out with NaCl, thorough drying of the salt cake between filter papers (to remove vasodilatins), fractionation with acetic acid, propylene glycol and methyl cellosolve, precipitation as the picrate, fractionation of the picrate with acetone, hot water, and acetic acid, and finally freeing of the hormone from the picric acid by precipitating it from a dry mixture of acetic and hydrochloric acids.

Data illustrative of the results obtainable are presented, and further methods of purifying the preparations are discussed.

[4] We are deeply indebted to Prof. Einar Hammersten and Dr. Gunnar Agren of the Karolinska Institut (Stockholm) for furnishing their very fine electrodialysis apparatus for these tests.

The potency has been increased to over 300 times that of the crude extracts, so that as little as 36 micrograms is sufficient to produce an accurately measurable response in a 20 kgm. dog of average responsiveness.

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INFLUENCE OF ALIMENTATION ON THE REGENERATION OF PLASMA PROTEINS FOLLOWING A SINGLE SEVERE NON-FATAL HEMORRHAGE[1]

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In previous communications (1, 2) in this Journal, changes in the red cell volume and the plasma proteins following an experimental bleeding were described. It was shown that the acute hypoalbuminemia which follows serious blood loss is corrected slowly, whereas the hypoglobulinemia returns to normal more rapidly. In these experiments the animals were not starved. In this report an attempt was made to study the effect of fasting as well as of various types of alimentation on the course of the regeneration.

Since our last paper (2) a few more observations have been described. Brown et al. (3) reported that following hemorrhage in man of 440 to 880 cc., hemodilution is detectable in one-half hour and continues for about 24 hours. If the blood loss is from an operation where there is extensive cutting of tissues, the hemodilution continues for 3 to 4 days. Beattie and Collard (4, 5) in anesthetized cats following single hemorrhages (20 per cent blood volume) obtained evidence that plasma proteins move into the blood stream within 2 hours after the hemorrhage. Considerable variations were observed in these experiments which the authors assume could be explained as being dependent upon the state of plasma protein stores outside the circulation.

METHODS. Mongrel dogs weighing between 8 and 15 kilos were used. They were allowed water but no food during the 18 hours preceding the experiment. Without anesthesia, except for the local use of novocaine when needed, the femoral artery was cannulated and the dog rapidly bled 35 to 45 cc. per kilogram of his body weight. No effort was made to affect the rate of blood flow from the cannula, but the process rarely required more than 2 minutes, after which the cannula was removed and the femoral artery tied off. The dog was then placed in a cage and allowed to have water ad lib. Except for immediate replacement of Ringer's solution in one group, the animal received no therapy until an hour after hemorrhage and then on the same hour on succeeding days. Heparinized blood samples were taken from the jugular vein or from the femoral vein in the leg opposite the one used for bleeding. These samples were centrifuged for 30 minutes at 3000 revolutions per minute. The plasma was analyzed for non-protein nitrogen and total nitrogen. For the former Nessler's reagent was used, for the latter a titrimetric micro-Kjeldahl procedure, involving steam distillation of the ammonia into a boric acid solution. For fractional determination of albumin the method of Campbell and Hanna (6) was employed. A few dogs died shortly

[1] Aided by a grant from the Commonwealth Fund.

after the hemorrhage, but in this report only those dogs surviving 72 hours are included.

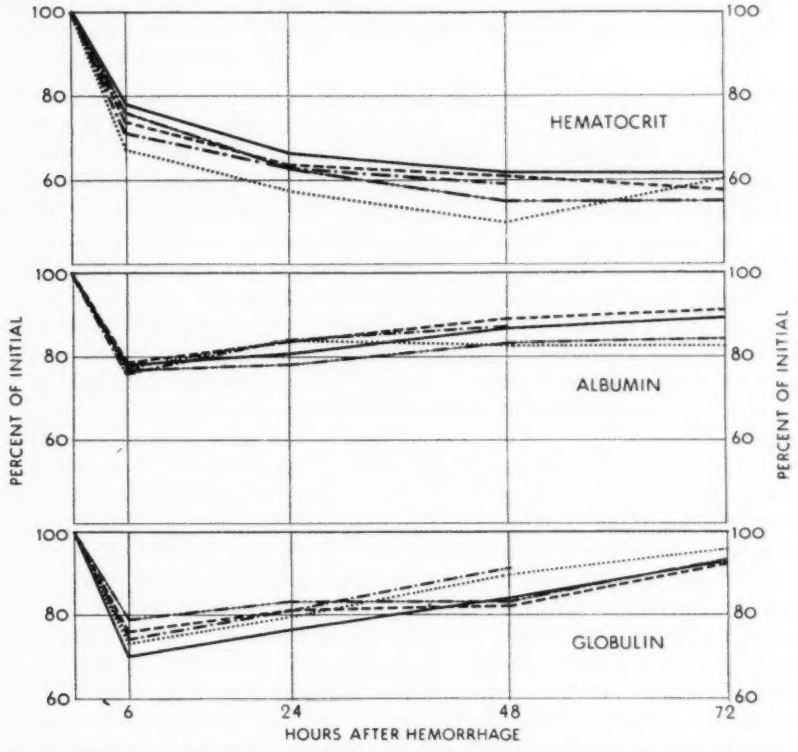

Fig. 1. Changes in hematocrit readings (red cell volume), plasma albumin and plasma globulin (expressed as percentages of initial which is represented as 100) during 72 hours following a single severe non-fatal hemorrhage, in five groups of experiments described in the text.

Symbols: group I, - - - -; group II, ——; group III, ...; group IV, —·—·—; group V, —··—··—

Note the pronounced and continued hemodilution shown by the drop in red cell volume and the fall and slow rise in globulin and albumin. Note that the five curves are quite similar, indicating the relatively slight influence of the various dietary intakes. The rise in globulin in these experiments is less pronounced than in previous experiments.

Five groups of experiments were performed as follows:

Group I. Ten dogs, 40 cc./kilo. hemorrhage; no repl; oral beef serum hydrolysate;[2] 75 cc./kilo. per day.

[2] Obtained through the courtesy of Dr. Warren M. Cox, Jr., of Mead Johnson & Company. The beef serum hydrolysate was enzymic as was Amigen, which is a similar hydrolysate obtained from casein.

Group II. Ten dogs, 40 cc./kilo. hemorrhage, no repl; oral 10 per cent glucose in n.s.s. 75 cc./kilo per day

Group III. Five dogs, 40–45 cc./kilo. hemorrhage; no repl; water ad lib, no food

Group IV. Six dogs, 35 cc./kilo. hemorrhage; repl. with Ringer's sol.; reg. kennel diet

Group V. Six dogs, 35–40 cc./kilo. hemorrhage; no repl; oral Amigen;[2] 75 cc./kilo. per day

It will be observed that only dogs in group IV received the usual kennel ration; all of them, however, were allowed water ad lib. Those getting oral therapy received the solutions by stomach tube. Vomiting occurred very rarely and when it did the dog was re-fed immediately. The beef serum hydrolysate and the Amigen were both 10 per cent solutions plus 10 per cent dextrose. To these was also added vitamin B in the form of Labco 20 cc. per 1000 cc. of solution. All gavage feedings were given in doses of 75 cc. per kilo per day. Therapy was given daily for 72 hours, then the animals were sacrificed.

RESULTS AND COMMENT. The values for hematocrit and plasma proteins were calculated as a percentage of the initial (pre-hemorrhage) value, which was put at 100. It will be observed that (see figure) among these various groups of dogs there is remarkably little variation in the behavior of the hematocrit values and the plasma proteins. The hematocrit readings fall rather abruptly at first and then more gradually throughout the whole 72 hour period. At 6 hours after hemorrhage the lowest values for plasma albumin and globulin are to be found. From the 6th to the 72nd hour, plasma proteins show slow but steady regeneration, the globulin curve rising a little more sharply than the albumin curve.

In our previous study (2) it was inferred that the rising level of serum albumin and the constantly falling hematocrit values immediately following a single severe hemorrhage meant that the blood volume was being restored with albumin-containing fluid. In the present experiment it is interesting to note that this mechanism is not affected by the therapeutic measures used, i.e., food, beef serum hydrolysate, Amigen, 10 per cent glucose. Moreover, the dogs receiving nothing but water exhibit the same ability to compensate for this acute protein loss as do the other dogs.

These negative findings are significant because they show that the body compensates for the acute protein loss from a single severe non-fatal hemorrhage in a manner which is unaffected by the presence or absence of exogenous sources of protein or carbohydrate, excluding, of course, protein introduced intravenously as substitution therapy, i.e., plasma, whole blood or serum. Not described herein are experiments in which solutions of amino-acids and hydrolyzed protein were given intravenously following a single severe non-fatal hemorrhage; the same negative results were observed as with oral administration. On the other hand, we have obtained evidence (7) which indicates that dogs *succumbing* to *repeated hemorrhages* are beneficially influenced by such injections; it would seem that the compensatory mechanisms following hemorrhage differ with the manner and severity of the loss of blood.

It is interesting to note the results of group III. It is well known that a non-protein diet will result, in 2 to 3 weeks, in hypoalbuminemia in dogs. Apparently

a non-protein diet even after a severe hemorrhage has little effect on the serum proteins of dogs for as short a period as 72 hours. Other workers (8) have found that amino-acids are beneficial in protein regeneration following hemorrhage, but these studies extended over a much longer period of time (weeks and months) than the experiments reported in this paper.

CONCLUSIONS

The presence or absence of exogenous sources of protein or carbohydrate alimentation does not influence the rapidity with which the body compensates for the acute plasma protein loss during the first 72 hours following a single severe non-fatal hemorrhage.

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THE EFFECT OF CHOLINE AND CYSTINE ON THE SERUM PHOSPHATASE AND HEPATIC DYE CLEARANCE OF DOGS MAINTAINED ON DEFICIENT DIETS

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In a previous communication (1) it was reported that dogs fed a protein-deficient diet had a reduced liver function as evidenced by an increased serum phosphatase and a decreased hepatic dye clearance. The changes in liver function produced by a protein-deficient diet could be restored to normal by an adequate protein intake.

There are numerous reports in the literature (2, 3, 4, 5) which show that choline administration tends to reduce or prevent the accumulation of fat which otherwise occurs in the livers of rats fed a high-fat, low-protein diet. It has also been shown (6, 7, 8, 9) that cystine administration increases the percentage of fat in the livers of rats maintained on a high-fat, low-protein diet. However, there is no information in the literature concerning the effect of these two substances upon the function of the liver as measured by dye clearance or serum phosphatase. The present study was undertaken to supply such information. In addition, data were obtained on the effect of a choline deficiency without a superimposed protein deficiency on the dye clearance and serum phosphatase. The effect of feeding methionine to protein deficient dogs was observed in a few experiments.

METHODS. Healthy mongrel dogs or puppies were used in all the experiments. These were maintained for several weeks on a normal kennel diet, during which time control values for serum phosphatase and hepatic dye clearance were obtained. The dogs were then fed one of the deficient diets for varying lengths of time. The composition of the diets used is shown in the table on page 643.

Diet I is the protein-deficient diet used in the previous study. Diets II and III are similar to the low-choline, peanut meal diet described by Engle (10). In II the B-complex was supplied by the six per cent yeast in the basal mixture. In III the crystalline vitamins were used. The peanut meal[1] was percolated with 65 per cent alcohol for twenty-four hours and then extracted five times with boiling 95 per cent alcohol. The casein was extracted three times with the same solvent.

With only a few exceptions the serum phosphorus, phosphatase and hepatic dye clearance were determined at two week intervals. Bodansky's method (11) was used for phosphatase and the Rose Bengal test of Stowe, Delprat and Weeks (12) with the modification described in the previous publication (1), was used to determine hepatic dye clearance. The dogs were weighed weekly. Total liver lipids were determined in duplicate on the whole fresh liver, using the method of Best, Channon and Ridout (13).

[1] Purchased from Darling Co., Chicago, Ill.

	DIET I	DIET II	DIET III
Lard	33%	25%	27%
Sucrose	55%	29%	31%
Salt mix	2%	4%	4%
Cellophane	5%	0%	0%
B-complex	Yeast 5%	Yeast 6%	Cryst. vitamins*
Protein	0%	Peanut meal 30%	Peanut meal 30%
		Casein 6%	Casein 6%

* The crystalline B-vitamins used were[2]: B_1, B_2, B_6, and calcium pantothenate (1 mgm./100 gram of diet), nicotinic acid, p-amino benzoic acid and inositol (10 mgm./100 gram of diet). One drop of Meade Johnson's percomorph oil was added to each 100 grams of diet. This supplied approximately 50 I.U. of vitamin D/kgm. and 350 I.U. of vitamin A/kgm. daily to adult dogs and double that amount to puppies.

The casein and peanut meal were extracted as described below.

The yeast used was Annheuser Busch Strain K.

All the diets were fed at a level of forty calories per pound of body weight for adult dogs and eighty calories per pound for puppies.

RESULTS. *Group I.* The effect of choline or cystine administration when fed at the beginning of a depletion period upon the serum phosphatase and dye clearance of adult dogs maintained on a diet practically free of protein (diet I).

a. Nine control animals were maintained on this diet and received no supplements. Five animals received diet I plus one-half gram of choline chloride daily in capsule form and six animals received diet I plus one gram of cystine daily throughout the experimental period. One animal was fed diet I, and given one gram of methionine daily for six weeks. At the end of that time the methionine was withdrawn and one-half gram of choline chloride was fed for two weeks.

b. Other dogs were fed choline or cystine supplements after they had been on diet I for varying lengths of time and had already exhibited increased serum phosphatase values and decreased rates of dye clearance. Several animals were fed $\frac{1}{2}$ or 1 gram of methionine daily after a period of protein depletion.

The differences in the serum phosphatase and hepatic dye clearance which were found in the twenty dogs in group I (a) with or without choline and cystine supplements are shown in table 1-a. The choline or cystine supplements which the treated animals in this group received were fed from the beginning of the depletion. The effect of choline during the first eight weeks is clearly seen when the serum phosphatase and hepatic dye clearance of this group are compared with the untreated group. At the end of this period the choline group's dye clearance had dropped from an original average of 104 $\pm$ 3.7 per cent of normal to 95 $\pm$ 5.8 per cent of normal. This is a relatively slight change. At the same time the untreated group's dye clearance had decreased from an original average of 99 $\pm$ 1.6 per cent of normal to 69 $\pm$ 6.2 per cent of normal. The

[2] The authors wish to thank Doctor Robertson of Merck & Co. for the crystalline B-vitamins, choline and methionine used in these experiments. The vitamin A and D was furnished through the courtesy of Mead Johnson & Co., Evansville, Ind.

choline group's serum phosphatase increased only 5.8 units while the untreated group's increased 16.6 ± 3.5 units.

The effect of cystine is best seen during the early weeks of the depletion. These animals exhibited a decrease in dye clearance during the first two weeks

TABLE 1-a

DOG	SERUM PHOSPHATASE					HEPATIC DYE CLEARANCE				
	Weeks									
	0	2	4	6	8	0	2	4	6	8
Diet I. Protein-free,* no supplements										
						per cent	*per cent*	*per cent*	*per cent*	*per cent*
C-1	1.0	3.5	7.5	9.8	17.3	106	109	78	82	86
C-31	1.2	4.1	5.8	8.7	10.9	95	80	76	77	78
C-32	1.2	5.8	8.7	10.9	12.2	103	97	87	85	90
8-E	2.1	6.6	14.8	17.6	32.6	102	93	86	80	50
9-E	1.3	2.4	4.6	5.9	5.7	100	100	87	77	76
10-E	1.3	11.3	14.8	15.0	32.7	92	67	69	87	53
12-E	2.0	7.0	21.1	30.8		100	86	63	48	
31	0.5	2.4	3.6	7.2	12.8	98	79	68	89	78
33	1.1	22.1	18.8	20.7	18.8	93	50	64	51	41
Average....	1.3	7.2	11.0	14.1	17.9	99	85	75	75	69
Diet I. Protein free; one gram of cystine daily										
17-S	1.0	13.1	30.0	23.1	38.9	118	69	67	69	50
20-S	1.7	19.9	30.5	23.1	46.8	114	69	60	54	43
21-S	1.1	10.2	29.6	20.5	27.8	126	76	62	69	59
22-S	1.4	13.6	27.1	22.7	32.2	130	102	88	41	83
23-S	1.0	4.1	9.3	9.6	12.7	102	80	67	61	65
30	1.9	7.4	21.4	10.4	13.0	108	72	79	61	72
Average....	1.4	11.4	24.7	18.2	28.6	116	78	71	59	62
Diet I. Protein free; one-half gram choline chloride daily										
10-S	3.5	4.2	6.6	6.5	8.4	106	114	90	89	77
14-S	0.1	3.5	4.0	6.2	4.7	90	109	118	96	99
15-S	1.8	6.2	6.3	7.5	9.1	109	103	84	103	105
16-S	2.5	7.4	11.8	12.1	12.2	111	110	102	111	108
34	0.6	2.2	1.5	2.5	3.0	102	106	100	79	86
Average....	1.7	4.7	6.0	7.0	7.5	104	108	99	96	95

* Except for the protein in the dried brewer's yeast.

from 116 ± 4.1 per cent to 78 ± 6.0 per cent. This decrease of 38 per cent is significantly greater than the two week decrease seen in the untreated animals which went from 99 ± 1.6 per cent to 85 ± 6.0 per cent, a decrease less than one half that seen in the cystine animals. At the end of eight weeks the cystine

animals had decreased 54 per cent as compared to the 30 per cent decrease found in the untreated dogs. The serum phosphatase of the cystine animals was 28.6 ± 5.7 units in contrast to the 17.9 ± 3.5 units found in the untreated group. In comparing the cystine and untreated groups it will be noted that the differences between them are most pronounced early in the experiment. The

TABLE 1-b

Protein-deficient dogs

DOG	LIVER FAT	SURVIVAL	FINAL SERUM PHOSPHATASE	FINAL HEPATIC DYE CLEARANCE	WEIGHT CHANGE
			No supplements		
	per cent	*weeks*		*per cent*	*lbs.*
C-1	12.0	14	36.2	50	−5
C-31	6.7	16	8.8	61	−3¾
C-32	14.4	12	35.0	41	−7
31	8.2	10	26.9	46	−6
33	27.7	12	31.6	40	−5
Average.....	13.8 ±2.7	12.8	27.7	48±	
			Choline supplement		
10-S	7.7	10	13.5	60	−1½
14-S	19.0	18	9.4	49	−6½
15-S	11.6	14	6.3	70	−7½
16-S	6.1	14	7.9	78	−6
34	5.4	10	4.3	107	−½
Average.....	10.0 ±2.5	13.2	8.3	73	
			Cystine supplement		
17-S	15.7	10	32.3	59	−5½
20-S	19.5	12	7.4	40	−5½
21-S	11.2	12	20.3	54	+½
22-S	14.7	12	26.7	27	−½
23-S	7.0	16*	9.9	53	+1
30	10.5	10	10.9	70	−2½
Average.....	13.1 ±1.8	12.0	17.9	51	

* Approximated.

cystine animals continue to exhibit decreases in dye clearance and increases in phosphatase, but the changes occur at a slower rate and eventually these values fall in the same range as the untreated animals whose values in the meantime have continued to drop.

Although all the animals survived longer than eight weeks this period of time was selected for the comparisons because soon after some of the animals developed varying degrees of anorexia. Serum phosphatase, liver lipids, and dye clearance

in dogs are all known to be affected by starvation (14, 15), consequently any values obtained after anorexia has developed become difficult to interpret. However, in order to obtain data on survival time all dogs (with the exception of four control dogs which were used to test other dietary supplements) were continued on the diet until they had developed practically complete anorexia. It was found that although the choline animals appeared better during the first eight weeks on the basis of the test used and also as judged by their appearance, they deteriorated rapidly after the eighth week and on the whole did not survive any longer than the control group. All three groups began decreasing their food intake about the same time. The average survival time of all the groups was between twelve and thirteen weeks. One of the factors which may have increased the variations among the individual animals in regard to survival time is coprophagy.

No control animals receiving adequate protein are included in this study because data on this aspect of the problem were presented previously (1) and gave adequate proof that diet I supplemented with 25 per cent casein protected dogs for as long as 30 weeks. No decreases in hepatic dye clearance were found, serum phosphatase remained normal, and liver lipids were low.

Since these experiments were planned primarily to study the changes in the phosphatase and dye clearance, it was impossible to control them in such a way as to make a satisfactory comparison of the liver fats in these animals. Nevertheless, the values found for liver lipids at autopsy are given in table 1-b along with survival time, final serum, phosphatase, dye clearance values, and weight changes. None of the differences between the cystine and untreated groups are at this time statistically significant. The choline group has significantly higher dye clearance and lower serum phosphatase but the liver lipids are not significantly lower.

The various experiments where cystine, choline or methionine supplements were fed after the animal had already been depleted are shown in table 2. The length of time the animal was maintained on diet I before the supplement was fed varied since the intention was to get a significant elevation in serum phosphatase and a decrease in dye clearance before giving one of the supplements. When choline was fed to these animals the serum phosphatase generally decreased and the dye clearance increased. Cystine usually had the opposite effect, causing a further rise in phosphatase and decrease in dye clearance. On one occasion the removal of the previously added cystine was followed by a large decrease in phosphatase, but no change in dye clearance, while in another instance its withdrawal was followed by an increase in dye clearance and only a slight decrease in phosphatase.

The depleted animals which were fed methionine gave variable results. In some cases this amino acid had the same effect as choline, in other cases it had no effect. The amount fed (one-half or one gram daily) was limited by the availability of the methionine and it seems reasonable to suppose that larger amounts fed for the same or greater periods of time would produce positive results. One animal[3] which received one gram of methionine daily from the

[3] Not shown in table 2.

beginning of a depletion responded in the same manner as a typical protein-deficient animal. At the end of six weeks his phosphatase was 17.3 units and his dye clearance had decreased from 111 per cent to 68 per cent. At this time the methionine was withdrawn and one-half gram of choline was fed. In one week the phosphatase had dropped to 5.2 units and the dye clearance had risen

TABLE 2

DOG	DAILY SUPPLEMENT TO DIET I	DAYS	PHOSPHATASE		DYE CLEARANCE	
			Start	End	Start	End
	The effect of choline on dogs already depleted of protein					
					per cent	*per cent*
5-D	½ gram choline	14	35.8	14.7		
1-E	½ gram choline	21	7.2	3.8		
2-E	½ gram choline	14	32.6	12.7		
3-E	¼ gram choline	14	21.5	14.8	79	95
4-E	¼ gram choline	14	32.0	17.6		
8-E	½ gram choline	14	23.5	21.1	56	76
10-E	½ gram choline	14	17.9	25.2	57	73
12-E	½ gram choline	14	22.1	21.7	61	70
	The effect of cystine on dogs already depleted of protein					
5-E	1 gram cystine	14	49.4	83.2	71	31
5-E	Cystine removed	14	83.2	44.9	31	34
9-E	1 gram cystine	14	5.2	15.9	63	33
9-E	Cystine removed	28	15.9	14.7	33	67
11-C	1 gram cystine	14	6.8	27.7	100	68
15-C	1 gram cystine	14	12.7	24.7	97	64
	The effect of methionine on dogs already depleted of protein					
5-D	1 gram methionine	14	26.9	14.7	68	64
5-D	½ gram methionine (immed. after above)	14	14.7	15.6	64	82
5-D	Methionine removed	14	15.6	31.4	82	66
33	½ gram methionine	14	12.7	18.8	51	40
33	Methionine removed	14	18.8	31.6	40	53
11-C	½ gram methionine	14	30.1	38.5	61	58
11-C	Methionine removed	14	38.5	30.6	58	39

to 90 per cent of normal. In two more weeks the animal had become moribund and was sacrificed. Nine per cent fat was found in his liver.

Group II. The effect of choline upon the serum phosphatase and hepatic dye clearance of puppies fed a diet containing adequate amounts of protein but low in methionine and choline.

A group of six litter-mate puppies was fed diet II. Three of these were given one-half gram of choline chloride daily. The others did not receive this supplement. Five of the animals developed pneumonia after four weeks on the diet,

but since the changes which occurred during the first two weeks showed significant differences they will be considered. The results obtained are shown in table 3. The definite differences obtained after two weeks on the diet demonstrate how rapidly changes occur and the sensitivity of the tests used. During

TABLE 3

Six litter-mate puppies. Peanut meal, choline-deficient diet

DOG	SUPPLEMENT	0 DAYS			14 DAYS		
		Weight	Serum phosphatase	Hepatic dye clearance	Weight	Serum phosphatase	Hepatic dye clearance
		lbs.		*per cent*	*lbs.*		*per cent*
79P	None	7¾	5.0	112	8	34.6	78
81P	None	7¼	4.1	118	6¾	16.8	66
82P	None	7¾	5.8	123	8¼	51.2	72
83P	Choline*	6¾	5.2	118	7¼	6.8	98
84P	Choline*	8½	4.9	120	9½	10.2	95
85P	Choline*	7½	5.4	123	8¼	7.8	89

* 0.50 gram choline chloride daily.

TABLE 4

Three litter-mate puppies. Peanut meal, choline-deficient diet

DAYS	DOG 60P			DOG 62P			DOG 64P		
	Diet II—no choline			Diet III—no choline			Diet III—½ g. choline daily		
	Weight	Serum pt.	Hepatic clearance	Weight	Serum pt.	Hepatic clearance	Weight	Serum pt.	Hepatic clearance
			per cent			*per cent*			*per cent*
0	10	4.9	141	10½	3.4	134	10½	5.6	126
14	10½	12.4	134	10	35.6	115	11½	17.1	100
28	11	31.5	85	9½	24.4	84	12½	9.6	116
49	11½	30.1	88	Died. Liver fat 16.2%			15	9.7	96
63	11½	30.1	88				15	9.7	105
	Changed to Diet III						Food allowance reduced to equal amt. which 60P ate		
77	11		82				15		104
84	10½	33.0	50				14½		
	Sacrificed. Liver fat 30.0%						Sacrificed. Liver fat 4.3%		

this two week period the animals receiving choline gained an average of three-fourths of a pound, or about 10 per cent of their original weight. The deficient puppies averaged no gain in weight. All of them consumed their diet completely during these two weeks. One of the deficient puppies did not become sick when the others did and was continued on the diet for eleven weeks. At the end of this time he was emaciated and inactive. His food consumption had

gradually decreased from one hundred and fifty grams daily the first three weeks to about thirty or forty grams daily at eleven weeks. He had lost about twenty-five per cent of his original weight, had a serum phosphatase of 45.8 units and a dye clearance 67 per cent of normal. One-half gram daily of choline chloride was then administered orally. During the following seven days his food consumption increased until he was eating two hundred grams daily. His phosphatase dropped to 19.8 units and in another week was down to 4.0 units. His hepatic dye clearance increased to 97 per cent of normal in seven days and to 107 per cent in fourteen days. By the end of three weeks of choline administration he was eating 300 grams daily, had gained four pounds and was healthy and active. Choline was then removed and ten days later he suddenly became ill and died. There was 19.0 per cent fat in his liver.

In table 4 is presented the data obtained on three other litter-mate puppies maintained on diets II and III. Here again the choline-deficient animals had a higher serum phosphatase (24.4 and 33.0 units), and a lower hepatic dye clearance than did the animal which received choline. The choline-fed animal had only 4.3 per cent fat in his liver while the deficient animals had 16.2 and 30.0 per cent respectively.

Discussion. It is apparent from the foregoing data that dogs receiving a high-fat, low-protein diet have a progressive impairment in the rate of removal of dye from the blood stream. The elevation of phosphatase is also progressive as the protein deficiency becomes more advanced. The anorexia which invariably develops in these animals may temporarily result in an increase in dye clearance and a decrease in phosphatase. The significance of the changes observed in the protein-deficient animals have been discussed previously (1).

The data presented in tables 1 and 2 indicate clearly that choline definitely aids in the maintenance of the liver function of the protein-deficient dog. That the beneficial effect of choline is temporary is also indicated by the eventual outcome of the experiments. Although at autopsy the liver lipids are in a slightly lower range than the untreated group and the dye clearance and serum phosphatase are closer to normal, the dogs had lost weight, developed anorexia and become moribund about the same time as the untreated animals. The animals in both groups had been practically devoid of dietary protein for more than ten weeks, and although the choline supplement may have prevented the infiltration of fat into the liver during the first eight weeks it could not prevent the loss of tissue protein from occurring during that time. This extensive loss of tissue protein results in numerous changes within the body which choline could not be expected to prevent. These changes and the anorexia associated with them cause the death of the animal, but should not be construed as casting doubt upon the effectiveness of choline during the earlier part of the experiment.

The results obtained on diets II and III indicate that a choline-deficiency in itself without a superimposed protein deficiency will cause a marked impairment in liver function in the normal puppy. In this case, however, oral choline supplements make the diet adequate and result in normal dye clearance and serum phosphatase. Schaefer et al have reported (16) that choline is essential for

the growing dog. The elevation in serum phosphatase which results from a choline deficiency in the puppy is very marked and rapid. The return to normal values which occurs when choline is fed is equally rapid. The data presented do not permit any conclusions as to the mechanism by which choline preserves liver function.

It is likewise apparent from the data that cystine early in the depletion increases the magnitude of the changes in dye clearance and serum phosphatase seen in the protein-deficient dog. The mechanism of the action of cystine is unknown. Cystine in large doses has been reported (17) to have a toxic effect upon the kidney. Griffith (5) has explained the action of cystine in accentuating the effect of a choline deficiency in growing rats as due to the acceleration in growth caused by cystine. This explanation is hardly applicable to the protein-deficient adult dog. It may be that the impaired liver function resulting from the protein deficiency impairs the means of utilization of cystine and thereby permits it to accumulate to the point of becoming toxic.

The experiments with methionine are inconclusive but indicate that on the weight basis this substance is less effective than choline in compensating for the protein deficiency.[4] This result is as one might expect if the methionine were serving as a source of methyl groups.

There does not appear to be a good correlation between the terminal fat content of a liver and its function as measured by dye clearance. Livers with a high function usually have a low fat content, but there are numerous instances of a low function and a low fat content of the liver. However, in such instances, histological examination frequently suggests other explanations for decreased dye excretion by the liver. This aspect of the problem will be considered in a later report.

SUMMARY

1. The decrease in dye clearance and the elevation of serum phosphatase caused by a protein-deficient diet are largely prevented in adult dogs for six to eight weeks by the daily ingestion of 0.5 gram of choline chloride. The effectiveness of choline was demonstrable only during the early weeks of the protein deficiency.

2. The changes in dye clearance and serum phosphatase caused by the protein-deficient diet are increased by the daily ingestion of one gram of cystine.

3. Choline-deficient puppies maintained on a diet low in methionine rapidly develop a marked increase in serum phosphatase and a decrease in dye clearance. Oral choline supplements will prevent or reverse these manifestations of impaired liver function.

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THE POLYPHASIC ACTION CURRENTS OF THE MOTOR UNIT COMPLEX

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It has been well established that the motor unit, as defined by Liddell and Sherrington (14) is a ". . . motor neuron axon and its adjunct muscle fibers . . . ."

Gilson and Mills (12) point out that a motor unit can be identified by a "simple rhythmic discharge of recorded spikes of essentially constant height and form," and that "this criterion of single unit activity has been considered acceptable by previous workers."

In previous studies (7), we occasionally saw action potentials which were characterized by the above description except that the wave form was complex instead of simple. They consisted of a series of deflections of varying voltage and duration. This led to the question of whether these complex forms represent *a*, discharges from single fibers in a single unit; *b*, discharges from various fasciculi in a single unit, or *c*, discharges from several units which are perfectly synchronized.

A subject of equal importance is the functional organization of units in an intact muscle. Smoothness of muscle contraction would seem to require that a single unit spread its effect to a certain degree; it seems possible, therefore, that a unit may be distributed through the muscle rather than be restricted to a small area.

We have been unable to find reports concerning the temporal relationships between spike complexes picked up by two or more leads, or describing the area from which a unit can be recorded, although Lindsley (15) mentions that "the movement of the electrodes a millimeter one way or the other often completely eliminates the response of a unit."

Since these questions are of fundamental significance in the analysis of the electromyogram, particularly in its growing use as a means of studying normal and pathological activities of the central nervous system and of muscle itself, further studies of complex muscle action potentials have been made.

One hundred and thirty-one records were made from 23 men and 1 woman. The muscles studied were the deltoid, biceps brachii, triceps brachii, brachialis anticus, extensor communis digitorum and various layers of the spinal extensors. In addition, records from a former series dealing with recordings from the spinal extensors by concentric electrodes have been reviewed.

APPARATUS AND METHODS. Action potentials were amplified by dual balanced amplifiers and observations were made with a loud speaker and a cathode ray oscilloscope. Records were made on bromide paper by means of oscillographs.

Three types of electrodes were used: 1, concentric electrodes; 2, bare hypodermic needles. These are likely to pick up potentials from several motor units, and they apparently permit some loss of voltage by leakage or cancellation; 3, hypodermic needles covered to within 2 mm. of the tip with enamel. This is a compromise between the highly selective coaxial electrode and the bare needle. It is

most efficient when paired with a distant intradermal electrode. If two of these electrodes are placed together, they become selective, like the coaxial, and often fail to pick up activity which can be demonstrated by moving one away. These electrodes were used in nearly all of the experiments. They were sterilized by heat or by soaking in 70 per cent alcohol.

With the subject in a copper screen room, a suitable area was selected and the skin overlying the muscle was cleaned with 70 per cent alcohol. It was then anesthetized by an intradermal injection of 2 per cent procaine hydrochloride. The usual procedure was to insert one needle in the desired spot and a second 10–15 cm. distant, the second being inserted intradermally and used as an indifferent electrode. The needles were connected to the amplifier grids. A skin electrode covered with electrode paste served as a ground lead. The subject then created enough tension to activate a motor unit near the electrode in muscle. If a good single unit could not be brought in easily, the needle was moved until a suitable location was found. This electrode (no. 3) and the indifferent (no. 4) were connected to the A amplifier (A or upper tracing on the records) and used throughout the experiment as a reference electrode. Four other needles were then inserted around the central needle, those in the vertical plane (long axis of the muscle) were usually 2 cm. above and below the reference electrode; those in the transverse plane (right angles to the long axis) were usually 1 cm. from the reference electrode. The vertical electrodes were numbered 2, above, and 6, below, those in the transverse plane were 1, left, and 5, right.

Measurements of the voltage and time recorded photographically from action currents were made as follows: Voltage was measured by comparing the height of the spikes with those produced by known inputs. Time was measured from the peak of the main spike on the A channel, which was taken as a fixed point. Measurements of all deflections were taken with a micrometer ocular and graphs were made from those data. For each record, 10 spikes were measured and the data given below represent average values.

Results. *The motor unit complex.* The muscle action potential of a single unit can be monophasic, diphasic, triphasic or polyphasic, as shown in figures 1 and 4, depending upon several factors. We have observed that all units in representative large muscles show distinctive patterns which vary in voltage and time with different electrode placements. The most common time intervals recorded ranged from less than 0.5 msec., where there is merely a hesitation or a notching, to 10–12 msec., where the two spikes are sharp and distinct.

The greatest disparity in time between spikes in one complex was seen in an experiment (fig. 4) where the time between the first and the last spikes of the complex was 22 msec. With a type 3 electrode, paired with an indifferent, picking up a single unit, a concentric electrode was inserted so that the tips of the type 3 electrode and the concentric were not more than 2 mm. apart. The first part of the complex was recorded simultaneously and with the same voltage on both. Twenty-two milliseconds later the concentric recorded an additional diphasic spike. The time between the first and last spikes thus recorded was constant in a series of photographs taken over a fifteen minute interval.

Augmentation and cancellation of the spike complex are demonstrated when two electrodes are in the field. When paired with the electrode out of the field

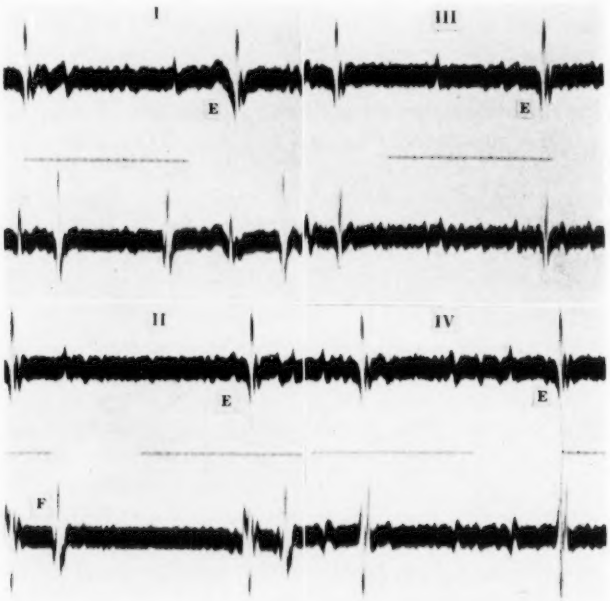

Fig. 1. Records taken with type 3 electrodes placed in the cross described above. The up swing is negative in all records. Time 0.25 sec.

Upper records: I–IV. electrodes 3 (in field) to 4 (out of field)

Lower records: I. Electrode 6 (2 cm. below 3 and in the field) to 4 (out of field).

Complex *E*—The single spike is nearly monophasic, an increase in amplification makes it triphasic, a decrease makes it purely monophasic. It occurs regularly 5.5 msec. before the main spike on the upper record.

II. Electrode 6 (2 cm. below 3 and in the field) to 3 (in field).

Complex *E*—The first negative spike on B is well ahead of the first negative spike on A and corresponds in time to the negative spike of E, lower record I. As 3 on B is in reversed position, in relation to the amplifiers, as contrasted to A, this large spike is obviously the pick-up at 3, while the early negative spike on B is from 6.

Complex *F*—The complex on the upper record is monophasic while on the lower it is polyphasic. The double negative spike and the notch in the second positive spike on B are examples of the complex wave form of the unit as described in the text.

III. Electrode 2 (2 cm. above 3 and in field) to 4 (out of field).

Complex *E*—The first and second negative spikes on B occur regularly 4.4 msec. and 6.6 msec. respectively after the large negative spike on the upper record. They correspond to the small spikes at the end of the complex on A in time but not in voltage.

IV. Electrode 2 (2 cm. above 3 and in field) to 3 (in field).

Complex *E*—The complex on B is a typical combination of the pick-up at 3 plus the pick-up on 2. 3 is in reversed position in relation to the amplifiers as contrasted to A.

each records a distinctive pattern. When they are inter-connected the temporal relation of the spikes of each discharge remains the same, with but minor varia-

tions. However, the voltages of the several spikes usually show marked change. In one experiment with two electrodes in the field, when the two were paired, a spike was recorded which appeared on neither when each was paired with an indifferent. In the same complex a spike, which was recorded with one electrode in the field, failed to appear when both electrodes in the field were paired as shown in figure 3.

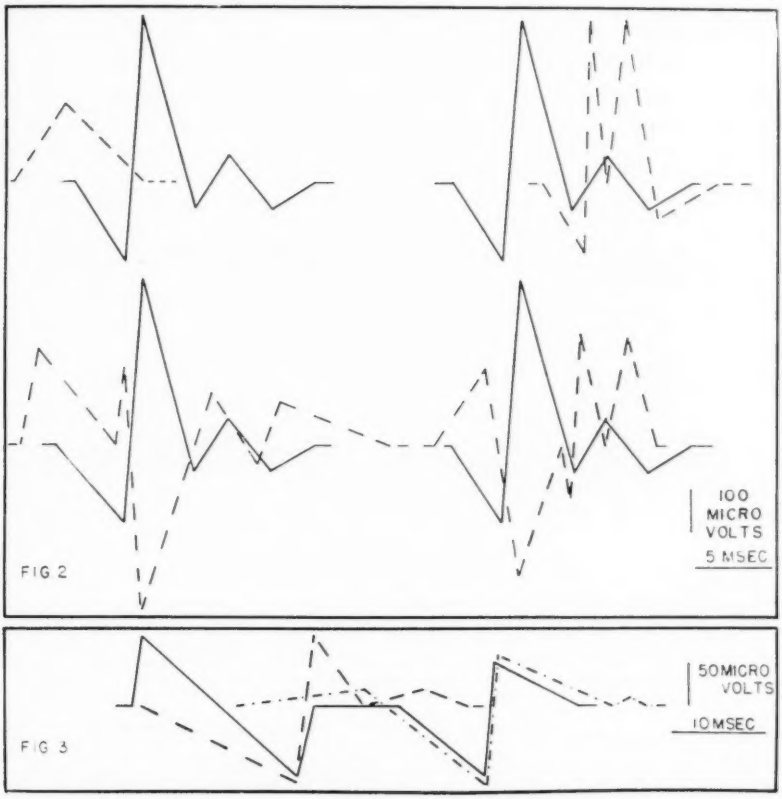

Fig. 2. Graphs representing the temporal and voltage relations of the E motor unit complex in figure 1; the upper left is I; lower left, II; upper right, III; lower right IV. The solid lines correspond to the upper records of figure 1 and the dashed lines to the lower records.

Fig. 3. Graphs representing the pick-up of two electrodes in a field. Solid line—electrodes interconnected. Broken lines—individual electrodes connected to indifferent electrodes.

Spatial delineation of the motor unit. Theoretically, it should be possible to ascertain the exact dimensions of the motor unit complex by moving the exploring electrodes back and forth until the area has been delimited. Practical difficulties, however, exist. The identity of the reference unit must be maintained at all times. If, through slight unavoidable electrode movement the pattern changes, the unit can no longer be identified positively as the original. In addition, after

numerous insertions and reinsertions of electrodes the subject frequently becomes tense and is unable to bring in the reference unit without having additional units contract.

In mapping the area from which a motor unit could be recorded, a contracting unit was located and electrodes were inserted as described. With the central reference electrode always on the A (upper) channel, all the others were connected in turn to the B (lower) channel. Some or all of the exploring electrodes picked up the activity. Electrodes in the field were moved outward, those out of the field were moved inward, until the limits were found.

In 52 placements 2 cm. from the reference electrode in the vertical plane, the unit was recorded 28 times and was not recorded 24 times. It was frequently recorded at distances less than 2 cm. and occasionally seen at greater distances.

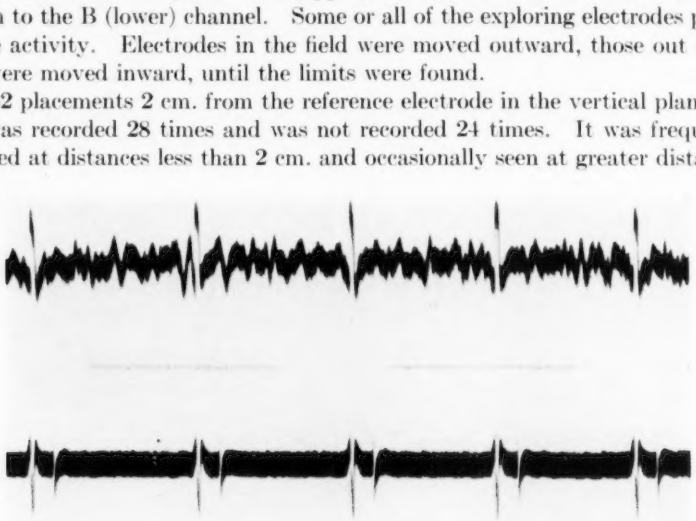

Fig. 4. A—upper record—electrode 3 (in field) to electrode 4 (out of field).
B—lower record—concentric electrode, about 2 mm. from tip of 3.
Time 0.25 sec.
Electrode 3 and the concentric electrode both recorded a triphasic complex with an almost imperceptible notching of the main spike; 22 msec. after this complex, the concentric electrode recorded a diphasic complex which did not appear on the 3 to 4 combination.

In the transverse plane, in 38 placements 1 cm. from the reference electrode, the complex was recorded 21 times and was not recorded 17 times.

While these distances (2 cm. above and below and 1 cm. to each side of the reference electrode) were close to the outer boundaries in most instances, no distinct configuration of the motor unit has been found. Nor was it possible to determine the configuration from its voltage. In one experiment the highest voltage at any lead was 140 μv; the vertical area of the pick-up was 7 cm. and the transverse 6 cm. In another, the reference electrode voltage was 500 μv with no pick-up 1 cm. to either side and 2 cm. below. The voltage recorded on different placements ranged from 5 to 1000 microvolts as measured on the highest spike in the complex. These figures are relative, for electrode movement of less than a millimeter may change the voltage as much as 100 per cent.

The data given above refer only to the horizontal plane. All electrodes were inserted to equal depths, but a few trials showed that all parts of a complex were

not necessarily picked up at a uniform depth. Partial withdrawal of an electrode frequently changed its pick-up. In one experiment a needle inserted to full depth (25 mm.) recorded 240 μv. At 22 mm. it registered 300 μv; at 17, 60 μv; at 12, 50 μv. Even more striking variations were found in penetrating resistant fascial sheaths; on one side no activity might be found, whereas 1–2 mm. farther, on the other side of the sheath, a spike of 200–300 μv could be recorded.

Time of propagation. In 24 placements in the long axis of the muscle where an action current was recorded on 2 electrodes, measurements of the propagation time from one electrode to the other were made. The electrodes were from 0.5 cm. to 7.0 cm. apart. The measurements represent meters per second and were reached by dividing the space interval between the electrodes by the time required for the action current to travel from one electrode to the other. They are as follows: 1.3, 1.6, 2.2, 2.5, 2.6, 2.7, 3.1, 3.2, 3.4, 3.6, 3.6, 4.3, 4.3, 4.9, 5.2, 5.3, 5.9, 6.1, 7.1, 7.1, 7.7, 7.7, 10.9, 12.5.

The spike complex recorded by concentric electrodes. In a previous series where we were concerned only with the presence or absence of motor unit activity, concentric electrodes were used exclusively. A review of twenty-five single motor units recorded with these electrodes revealed that in 21 instances the spikes were di- or triphasic. In only 4 were they polyphasic. To verify this we made additional records with a concentric electrode. The wave form was altered by changing the position of the electrode (e.g., by rotation).

Discussion. Three possible explanations for the polyphasic nature of the complexes recorded from motor units have been suggested. These are: *a*, action currents from single muscle fibers; *b*, action currents from several motor units firing synchronously; *c*, action currents from the fasciculi of a single motor unit.

Neither *a* nor *b* offers a sound explanation for polyphasic complexes. When the electrodes are compared in size with the muscle fibers, it is found that a large number of muscle fibers (diameter ca. 50 micra) would always be in contact with the electrode (diameter 450 micra). Therefore, if the individual muscle fibers were the source of the spikes, there would be more spikes than are actually found.

Many investigators have reported their observations on the frequency of motor units and there seems to be general agreement that two or more motor units do not fire synchronously except for extremely brief periods. To accept the theory that polyphasic action currents are due to a synchronization of motor units would require rejection of the concept of motor unit function which has been built upon the original observations of Adrian and Bronk (1, 2) and Denny-Brown (6).

Considerable evidence indicates that the polyphasic complex is due to *c*, action currents from the fasciculi of a single motor unit. Although little is known about the histology of the motor unit in large muscles, it has been shown to consist of a motor neuron and muscle fibers varying in number from 120 to 160 (9, 4, 16). Since muscle fibers are grouped into fasciculi containing about 20 fibers (3) and since the total number of fibers in a motor unit varies (11), it is

apparent that although the number cannot be fixed with certainty, there are at least several fasciculi in each unit.

Cooper (5) states that in the frog tenuissimus chains of fibers contract along the whole length of the muscle at all strengths of contraction.

The electromyographic data described above reinforce the idea that the motor unit is broken up into smaller components and that they are scattered. The spike complex usually registers as one or two strong action currents plus a number of weaker ones. We can infer from this that the electrode is near or in contact with one or two fasciculi and relatively distant from the rest.

Although there are no reports concerning the rate of conduction of impulses over the very small distal motor axon branches in the human neuromuscular system, the work of Erlanger and Gasser (10) permits the assumption that it is fairly slow, as is the velocity of the muscle action current. Katz and Kuffler (13) estimate the latter to be 2.–2.5 m./sec. in the frog sartorious, and Eccles and O'Connor (8) estimate rates of 2.85–4.8 m./sec. for cat soleus, 4 to 7 m./sec. for cat peroneus. Hence, the time difference of two action currents recorded on one electrode in the same unit can be accounted for by differences in the length and diameter of the involved axon branches and the spatial relation of the electrode to the fasciculus and its axon branch. In complex patterns when two leads record spikes in the same temporal relationship but with different voltages it is apparent that the spatial relation of the two to the fasciculi was similar except for slight differences in electrical contact.

There are interesting parallels between the polyphasic complexes we have seen and the polyphasic and notched complexes reported by Katz and Kuffler (13) in their studies of multiple motor innervation of the frog's sartorious muscle. In figure 3 of their paper, A2 and A3 show polyphasic wave forms representing the action potentials initiated first by nerve impulses over the pelvic branch, and a few milliseconds later by impulses from the tibial branch. In B2 and B3, with the pelvic branch cut, the first half of the complex wave fails to appear as the electrodes pick up one instead of two sets of action potentials.

Another interesting parallel is seen in figures 3A and B in a report of Lindsley (15). Although he did not discuss wave form, these figures show two negative and two positive spikes in clearly defined single units.

The demonstration of two or more spikes in a single motor unit implies that the unit is composed of discrete parts, and signifies that electromyographic studies must be interpreted on the basis of the functional capacity of the anterior horn cell with its motor neuron and of each of the muscle groups which it activates. It is apparent that both must be considered in any analysis of normal or pathological function of the final common path.

The present attempts to discover an exact pattern for the motor unit have been ineffective. No quantitative data are available concerning the distance an electrode can be located from a group of contracting fibers and still retain electrical contact. Our observations suggest that this distance is greater in the long axis of the muscle. This is perhaps due to greater conductivity of the fibers in that direction, but since fascial sheaths are in that plane, as insulators they doubt-

less restrict the pick-up in the transverse plane. Hence, these questions of conductivity and of interposed tissue must qualify the observations that the unit frequently occupies an area longer than its longest fiber and considerably wider than the combined diameters of its fibers. However, it has been apparent that an adequate sampling of an area 4 cm. square for muscle activity can be made by placing four type 3 electrodes, each paired with an indifferent, at the corners of a 1 cm. square.

In general it can be stated that the flow of action currents in a single unit is roughly parallel to the long axis of the muscle fibers and that it is progressive from one end of the unit to the other. We have never seen an instance where the action potentials of a single unit as recorded on electrodes 2 and 6 were both before or both after the complex as it was recorded on electrode 3.

The observations concerning propagation of the action current are qualified by the consideration of distance between active fibers and recording electrode, by the possibility that the two recording electrodes might be in different fasciculi (in one experiment the action current occurred simultaneously on 2 electrodes 2 cm. apart), and because precise electrode placement is not possible in unexposed muscle. Even with these qualifications, it seems safe to assume that the rate of propagation of the muscle action current is between 2.5 and 8.0 meters per second. These observations are in general agreement with previous reports (8, 13).

SUMMARY

1. Mono-, di-, tri- and polyphasic action currents from single motor units have been recorded, the pattern of the discharge being influenced by the type and location of the electrodes.

2. The area from which a motor unit can be recorded varies from 2 to 7 cm. vertically and 1 to 6 cm. horizontally; fascial sheaths probably act as dielectrics and limit the fields.

3. The evidence indicates that the components of the motor unit discharge originate in the several fasciculi which compose the motor unit.

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THE REACTIVITY OF AUTONOMIC MEDULLARY CENTERS UNDER CONDITIONS OF RESTRICTED BRAIN CIRCULATION

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The measurement of blood flow through the brain under various experimental conditions has revealed an amazing degree of adaptability of the brain circulation. Rein and Schneider have shown that on clamping of one carotid artery, the blood flow in the remaining carotid is increased about one hundred per cent. Successive ligation of the contralateral carotid and of both vertebral arteries increases the blood flow through the interna carotid more than twice. These reactions appear to be responsible for the remarkable fact that even the clamping of both carotids does not cause any permanent effect on the cerebral potentials of the cat (Bremer). Asenjo observed that only the clamping of both carotids and one vertebral artery definitely reduced the brain potentials. The reactivity of the autonomic centers in these conditions of restricted brain circulation has not yet been studied adequately. Since the vasomotor as well as the respiratory center reacts with great precision to slight alterations in the oxygen and carbon dioxide tension of the blood, it seemed of interest to investigate the reactivity of these centers when one or more of the main arteries are ligated temporarily.

METHODS. The experiments were carried out on 43 dogs anesthetized in most instances with 70–100 mgm./kgm. chloralosane intravenously. In some animals sodium amytal (55 mgm./kgm. intraperitoneally) was used. Blood pressure and respiratory rate were recorded in the usual manner. The respiratory minute volume was determined by means of a spirometer. The respiration was either spontaneous or artificial after a pneumothorax had been made. Gas mixtures ranging between 4.5 and 12.5 per cent oxygen and 3.2 and 15 per cent carbon dioxide were inhaled from Douglas bags.

RESULTS. I. *Observations on the effect of anoxia and hypercapnia on blood pressure and respiration of "denervated"*[1] *dogs under conditions of restricted brain circulation.* In order to study the reactivity of the vasomotor and respiratory centers to changes in the oxygen and carbon dioxide tension of the blood under the conditions of restricted brain circulation it was deemed advisable to eliminate the pressor and chemo-receptors which modify the reactivity of these centers. It was found in such preparations that the blood pressure response to inhalation of gas mixtures low in oxygen did not show any significant alteration in the fall of the blood pressure after one carotid artery or one or both vertebral arteries had been clamped. In the light of the experiments reported below, this suggests that no significant disturbance in brain circulation is induced under these conditions.

[1] Denervation of the sino-aortic area.

If, however, both carotid arteries are ligated, the reactivity of the respiratory and vasomotor centers is altered significantly. Figure 1 shows that the respiratory response to carbon dioxide is definitely increased under these conditions, although the clamping of the carotids as such does not change the respiratory minute volume. Furthermore, in experiments involving the inhalation of gas mixtures low in oxygen it is found that the fall in blood pressure which occurs in the "denervated animal" in anoxia is greatly increased after bilateral clamping of the carotids (table 1). In the experiments involving the use of oxygen-nitrogen mixtures a constant respiratory volume was maintained by artificial

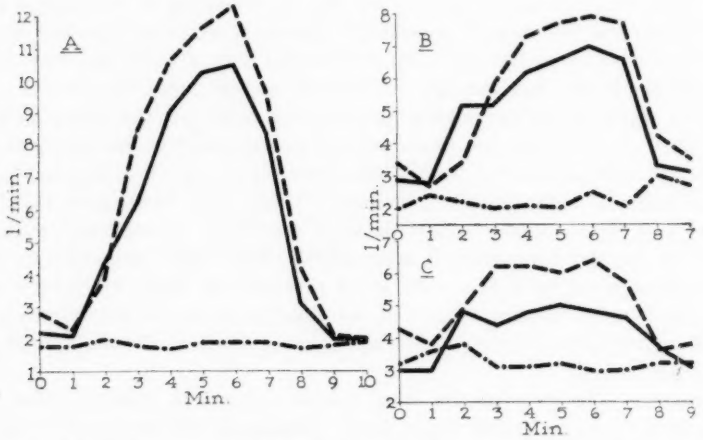

Fig. 1. A. Effect of 5 per cent CO_2 on the respiratory volume during clamping of both carotid arteries. Dog 38, 3/28/40, 9.1 kgm., 70 mgm./kgm. chloralosane intravenously. —·—·—, clamping of carotids between 1 and 9 while dog inhales air.————, inhalation of 5.4 per cent CO_2 from 1 to 6. — — — — inhalation of 5.4 per cent CO_2 from 1 to 6 while carotids are clamped from 1 to 9.

B. Effect of 5.4 per cent CO_2 on the respiratory volume of a "denervated" dog while the carotids are clamped. Dog 44, 4/22/40, 10 kgm., 70 mgm./kgm. chloralosane intravenously.

C. The effect of 3.2 per cent CO_2 on the respiratory volume of a "denervated" dog while the carotids are clamped. Same dog as in figure 1, A.

respiration since it was known from the work of Gellhorn and Lambert that the diminution or failure of respiration after elimination of the carotid sinus nerves may secondarily cause a fall in blood pressure. Gellhorn and Lambert's interpretation that the fall in blood pressure occurring in denervated dogs under conditions of anoxia is due to a depression of the vasomotor center appears to be applicable to these experiments. The augmented fall in blood pressure in response to anoxia suggests that the vasomotor center is more susceptible to anoxia after the carotid arteries have been clamped than under normal conditions.

It is interesting to note that this reaction may be observed also when in an animal in which both vertebral arteries had been permanently ligated the carotid

arteries are clamped temporarily. It is then found that under conditions of anoxia the fall in blood pressure observed on inhalation of 5 per cent oxygen is greater after the two carotid arteries had been clamped.

Experiment 4 of table 1 illustrates the effect of 6.2 per cent oxygen on the blood pressure of a "denervated" dog before and after ligation of the carotid arteries under conditions of spontaneous respiration. Here, likewise, a greater fall in blood pressure is observed under conditions of restricted brain circulation. In view of our earlier studies this reaction may, at least in part, be due to more rapid failure in respiration after the carotids have been ligated.

From these experiments it seems to follow that ligation of one carotid artery or of both vertebral arteries is fully compensated and does not lead to any altera-

TABLE 1

Effect of restriction in brain circulation on the blood pressure response to anoxia in dogs with denervation of the sino-aortic area

NO.	GAS MIXTURE	DURATION OF ANOXIA	EFFECT OF ANOXIA ON BLOOD PRESSURE						
			Without clamping		With clamping		Without clamping		
					of both carotid arteries				
				Diff.		*Diff.*		*Diff.*	
1	5% O_2	25 sec.	114–96	18	120–86	34			
					123–90	33			
2	6.2% O_2	45 sec.	92–73	19	104–76	28	88–72	16	Pneumothorax and artificial respiration
					98–74	24			
					95–72	23	84–74	7	
3	4.5% O_2	60 sec.	130–115	15	170–115	55	120–100	20	
4	6.2% O_2	5 min.	125–85	40	145–65	80	Spontaneous respiration		

tion in the reactivity of medullary centers when tested with gas mixtures low in oxygen or rich in carbon dioxide.

However, bilateral ligation of the carotid arteries leads to a disturbance in brain circulation which is revealed by the increased response of medullary centers to anoxia and carbon dioxide.

II. *Effect of restriction in brain circulation on the blood pressure and respiratory response to anoxia and carbon dioxide excess in normal and vagotomized animals.* Since ligation of the carotids of "denervated" dogs resulted in a greater depression of the vasomotor center to anoxia than was seen after restoration of the normal circulation, one might expect that in normal and vagotomized animals the pressure response to anoxia would be diminished under these conditions. However, the result is quite different. As figure 2 shows, ligation of the carotids greatly increases the pressure response to anoxia. Since anoxia depresses the vasomotor center more after the ligation of the carotids as shown by the experiments on "denervated" animals described in the preceding paragraphs, the

intensification of the pressure response must be due to other than central factors. Considering that in normal animals the ligation of the carotids greatly alters the reflexes mediated by the buffer nerves, it is probable that they are responsible for the alteration in the blood pressure response. The lowering of the pressure

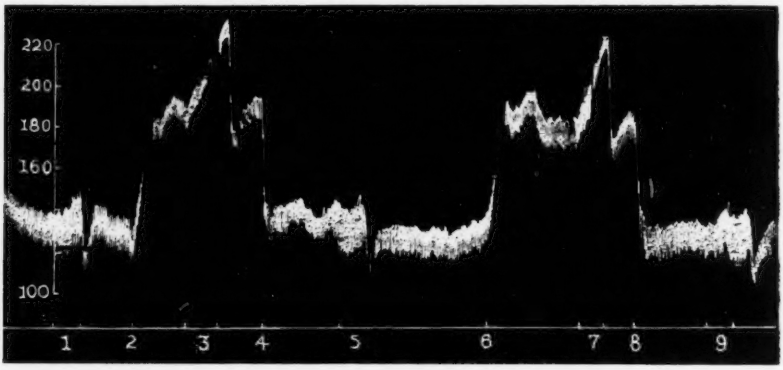

Fig. 2. The effect of 6.2 per cent O_2 inhaled for 1 minute on the blood pressure before and during restriction of brain circulation (clamping of both common carotids). Dog 9, 12/7/39, 8.2 kgm., chloralosane 100 mgm./kgm. intravenously. Bilateral vagotomy. *1*, *5* and *9*: 6.2 per cent O_2 under control conditions; *3* and 7: 6.2 per cent O_2 while both carotids are clamped (from 2 to 4 and 6 to 8 respectively).

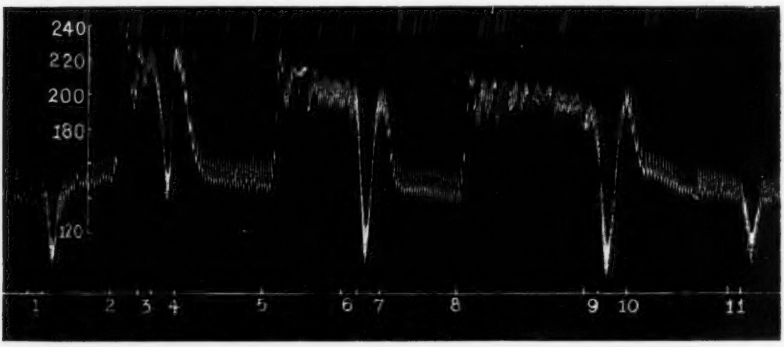

Fig. 3. Effect of 15 per cent CO_2 on blood pressure before and after clamping of both common carotids. Dog 29, 3/1/40, 7.6 kgm., 70 mgm./kgm. chloralosane intravenously. *1*, *11*, 15 per cent CO_2 inhaled for 30 sec. under control conditions. *3*, *6* and *9*, the same but carotids are clamped (from *2* to *4*, *5* to 7 and *8* to *10* respectively).

in the carotid sinuses which follows the ligation of the carotid arteries central to these structures is similar in its effect to that seen after removal of the pressure receptors. If this is the case, any factor which either raises or lowers the blood pressure should produce greater alterations in blood pressure after the carotid arteries had been clamped. It was, indeed, shown that the blood pressure rise following inhalation of carbon dioxide was also greatly exaggerated. It is of

interest to note that in several animals in which either anoxia or carbon dioxide caused a fall in blood pressure instead of a rise, this fall was greatly enhanced after the carotid arteries had been clamped (fig. 3).

It may be added that ligation either of one carotid artery or of both vertebral arteries did not alter the blood pressure response to anoxia.

Experiments on the effect of the inhalation of carbon dioxide under conditions of restricted brain circulation caused similar effects in normal and vagotomized dogs as have been described for animals deprived of the sino-aortic receptors (fig. 1). It was found that carbon dioxide had a greater effect on respiration after ligation of both carotid arteries. Since carbon dioxide stimulates the respiratory center as well as the chemoreceptors, it is not surprising to find that the reactivity of normal and "denervated" animals is similar. The increase in respiratory response to carbon dioxide after ligation of both carotid arteries varied considerably in different experiments but was distinct in all animals independent of the presence of the buffer nerves.

The increased level of the blood pressure together with the augmented blood pressure and respiratory response to anoxia and hypercapnia represents an adjustment reaction which tends to restore an adequate blood supply to the brain in spite of the elimination of both carotid arteries.[2]

CONCLUSIONS

The reactivity of the vasomotor and respiratory center under conditions of restricted brain circulation is determined by comparing the effects of anoxia and hypercapnia on blood pressure and respiration before and after ligation of one or more carotid and vertebral arteries.

It is found that neither ligation of one carotid or of one or both vertebrals alters the blood pressure response to anoxia.

Ligation of both carotids leads to an increased blood pressure response to gas mixtures low in oxygen in normal and vagotomized animals. After denervation of the sino-aortic area the blood pressure falls more rapidly during anoxia after both carotids had been ligated indicating an increased sensitivity of the vasomotor center to anoxia under conditions of restricted brain circulation.

The respiratory response to hypercapnia is increased after bilateral ligation of the carotids. This effect present in normal and vagotomized animals as well as in dogs in which the sino-aortic area had been denervated signifies an increased sensitivity of the respiratory center to hypercapnia.

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[2] For further discussion cf. Gellhorn (1943).